PATHOPHYSIOLOGICAL ASPECTS OF GLUCAGON METABOLISM IN DIETARY OBESE SHEEP

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

INTRODUCTION

Twenty percent of the American population is obese (56) Obesity predisposes individuals to develop metabolic diseases such as noninsulin-dependent diabetes mellitus (NIDDM). Eighty five percent of NIDDM patients are obese (158).

Obesity, insulin-dependent diabetes mellitus (IDDM) and NIDDM are characterized by impaired blood glucose homeostasis. Blood or plasma glucose level is controlled mainly by the two pancreatic hormones, insulin and glucagon. Insulin promotes glucose uptake and utilization in tissues (e.g., muscle) while glucagon enhances hepatic glucose output that serves to increase the plasma glucose level. An imbalance between insulin and glucagon will affect the plasma glucose concentration.

Elevated plasma insulin concentration (hyperinsulinemia) and defective insulin action (i.e., insulin resistance) in the insulin target tissues are common in obese humans and animals. However, whether or not plasma glucagon level is altered by obesity is not clear.

Reports on the effects of obesity on glucagon metabolism are conflicting, perhaps because a method for accurate measurement of plasma glucagon has not been well established. Quantification of plasma glucagon by RIA is complicated by the presence of large molecular weight plasma proteins that express glucagon-like immunoreactivity. In addition to the technical difficulties associated with measuring plasma glucagon, study

of metabolic defects in obesity oftimes is limited by the availability of obese subjects or an obese animal model. The etiology of obesity also may be an important determinant of how obesity affects glucagon metabolism in humans.

With access to an established obese animal model (102), this study was able to investigate glucagon metabolism in dietary obese sheep. Dietary obese sheep suffer metabolic defects that are similar to those found in obese humans. Obese sheep are hyperinsulinemic, hyperglycemic and insulin resistant. The dietary obese sheep should be an unique animal model for study of obesity simply because the cause of obesity is known. Variables such as diet type, energy intake, environment, age and sex of the animal are controlled in lean and obese sheep.

The overall objective of this study was to identify the pathophysiological changes in glucagon metabolism in dietary obesity. The specific objectives of this study were: 1) to establish a reliable method to quantify plasma glucagon concentration, 2) to determine the whole-body kinetics of glucagon in lean and obese sheep, 3) to determine how obesity affected the molecular profile of plasma immunoreactive glucagon under basal conditions and after arginine-induced rises in plasma IRG concentration, and 4) to determine if α-cell responsiveness to arginine stimulation differed in lean and obese sheep.

Chapter II

LITERATURE REVIEW

INTRODUCTION

Insulin was the first and glucagon was the second pancreatic islet hormone to be discovered. Murlin et al (114) unknowingly identified glucagon in 1923 when they reported that aqueous extract of the dog pancreas contained a hyperglycemic-glycogenolytic factor (HGF). It was this contaminant in the early insulin preparations that produced a short period of hyperglycemia in insulin-treated patients. This observation enhanced interest in glucagon research. Sutherland (154) and Heard (66) in 1948 demonstrated the presence of HGF activities in extracts of pancreas, mucosal cells of small intestinal, and gastric mucosa in several species, but pancreatic tissue contained the greatest amount of HGF.

Glucagon was first purified from pancreas and was identified as a peptide hormone by Staub (151) in 1953. The amino acid sequence of glucagon was reported by Bromer in 1957 (28), and glucagon was first measured in plasma by Unger (159) using radioimmunoassay (RIA).

Relative to studies on insulin metabolism, glucagon research has proceeded slowly. However, it is known that glucagon is synthesized by the preprohormone pathway and that proglucagon contains multiple bioactive peptides (70, 78). The physiological significance of most proglucagon gene products still remains unclear but knowledge about

regulation of glucagon secretion and glucagon action at the cellular and molecular level is growing (70, 78, 91).

GLUCAGON SYNTHESIS

Glucagon Structure

Glucagon is a linear polypeptide hormone that contains 29 amino acids; it has a molecular weight of 3,485 daltons (28; Figure 1). The amino acid sequence of glucagon is identical in most mammalian species (153). Glucagon is classified as a member of the secretin family of hormones because of its homologous structure to secretin, vasoactive inhibitory peptide (VIP) and gastric inhibitory peptide (GIP) at amino acids 6, 10, 13, 19, 22, 23, 25, 26, 27 (158).

NH_2 - His - Ser - Gln - Gly - Thr - Phe - Thr - Ser - Asp - Tyr - Ser - Lys - Tyr - Leu - Asp - Ser -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Arg - Arg - Ala - Gln - Asp - Phe - Val - Gln - Trp - Leu - Met - Asn - Thr - COOH
17 18 19 20 21 22 23 24 25 26 27 28 29

Figure 1. Amino acid sequence of glucagon (28).

Proglucagon Gene

Glucagon is synthesized as a 180 amino acid preprohormone which has a molecular weight of 12,000 daltons. In most mammalian species, including human and sheep, the proglucagon gene is expressed in alpha cells of the pancreas and mucosal cells of the small intestine (32, 41, 110, 119). In both the pancreas and the gut, the signal sequence of preproglucagon is removed as the proglucagon protein enters the rough endoplasmic reticulum (RER). Subsequently, part of the RER membrane is pinched off and forms

microvesicles which contain the proglucagon protein. Tissue specific cleavage of the proglucagon starts in microvesicles and continues after the microvesicles have fused with the Golgi apparatus (65). Bioactive glucagon is packaged as a tri-molecular crystal in secretory granules of the cytosol and is secreted from alpha cells by the process of exocytosis (158).

Tissue Expression of Proglucagon Gene

Pancreatic and intestinal preproglucagon mRNAs are identical in humans (119). Different peptides are secreted by the pancreas and intestine because multiple bioactive peptides can produced from proglucagon. Post-translational processing of progiucagon is tissue specific (48) as shown in Figure 2.

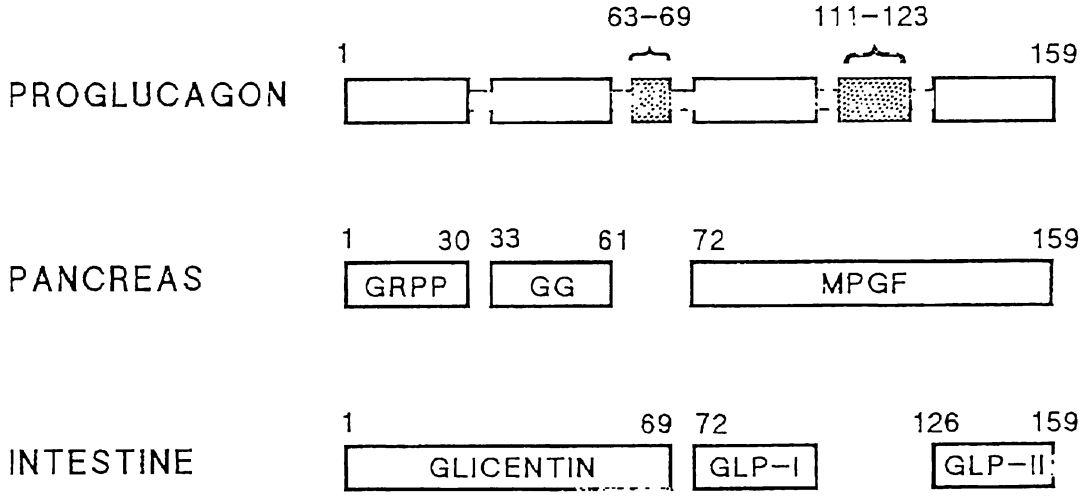

Figure 2. Schematic structure of progiucagon protein and its tissue-specific processing in pancreas and intestine. The numbers above the boxes correspond to the numbered residues within proglucagon. Solid boxes represent Lys-Arg or Arg-Arg connecting peptides and these are the sites for enzymatic cleavage of proglucagon. Abbreviations are GRPP, glicentin-related pancreatic peptide; GG, glucagon; MPGF, major proglucagon fragment; GLP-1, glucagon-like peptide 1; GLP-2, glucagon-like peptide 2.

Glucagon (3,485 Da) is the major secretory product derived from proglucagon in the pancreatic alpha cells. Glucagon is also found in gastric mucosal cells of dogs (146) and humans (16) but little or no glucagon is secreted by these cells (70). Pancreatic glucagon is responsible for the endocrine regulation of hepatic glycogenolysis and gluconeogenesis.

Bioactive fragments derived from proglucagon gene expression in the gut include glicentin and the truncated forms of glucagon-like peptide-1 (GLP-1) named GLP-1_{7-36} amide and GLP-1_{7-37} (47, 122). Glicentin and glucagon-like peptide-1 (GLP-1_{7-36}) amide and GLP-1_{7-37} are the major bioactive fragments derived from proglucagon that are secreted by the intestinal α-cells. Secretion of glicentin, GLP-1_{7-36} amide and GLP-1_{7-37} is induced by ingestion of food (47, 48) and intraluminal glucose absorption (95).

The physiologic function of glicentin is not well identified. Glicentin may function as an additional source of glucagon, because glicentin includes the entire structure of glucagon (70). However, it is also possible that glicentin may act as an antagonist of glucagon action in hepatocytes (70).

Glucagon-like peptide-1_{1-37} has no known metabolic effects in mammals, but its two shorter forms of GLP-1_{7-36} amide and GLP-1_{7-37} may act as an incretin (60, 124). GLP-1_{7-36} amide and GLP-1_{7-37} secreted by the intestine in response to nutrients in the intestinal lumen act in an endocrine manner to inform the pancreatic beta cells of the amount and composition of the upcoming nutrient absorption (60, 125). Support for this concept comes from reports showing that postprandial level of GLP-1_{7-36} amide level was significantly increased in humans.(60, 86). Additionally, infusion of GLP-1_{7-36} amide under euglycemic condition, raised the plasma concentrations of insulin (60).

REGULATION OF GLUCAGON SECRETION

Humoral Regulation

Amino Acids. Plasma glucagon concentration is significantly increased after consumption of a protein meal in humans (81). Apparently, this rise in the plasma glucagon level is in response to a rise in the plasma concentration of amino acids derived from the ingested protein. In vivo studies in humans (20, 73, 185), dogs (35, 134) and sheep (87) showed that intravenous administration of selected amino acids increased plasma glucagon concentration. Similarly, in vitro studies also showed that arginine increased the secretion of glucagon by rat islets (29, 156).

Most, but not all, amino acids are potent glucagon secretagogues. The potency of various amino acids in stimulating glucagon secretion has been studied in dogs (134) and sheep (87). Among the 20 amino acids tested, asparagine, glycine and phenylalanine were very strong glucagon secretagogues in dogs, whereas alanine, glycine, serine and arginine were the most potent of the 17 amino acids tested in stimulating glucagon secretion in sheep. Both studies showed that intravenous injection of valine, histidine, leucine and isoleucine did not increase plasma glucagon concentration.

The structure-function basis responsible for the different potencies of amino acids in stimulating a rise in the plasma glucagon level is not known. No significant relationship between the structure or size of the R-group and potency of an amino acid as a glucagon secretagogue has been shown (134). However, it is remarkable that in the studies by Rocha (134) and Kuhara (87) that the five most potent glucagon-secreting amino acids were gluconeogenic (Figure 3). Physiologically these amino acids stimulate glucagon secretion which in turn promotes the incorporation of these gluconeogenic amino acids into glucose in the liver. On the other hand, valine, leucine and isoleucine,

which have no effect on plasma glucagon level, are substrates for both the TCA cycle and the ketogenic pathway. That these ketogenic amino acids do not increase glucagon level may be an advantage, because this would prevent the development of ketosis due to inappropriate hyperglucagonemia. Therefore, the ability of individual amino acid to stimulate glucagon secretion may depend on whether they are gluconeogenic or ketogenic.

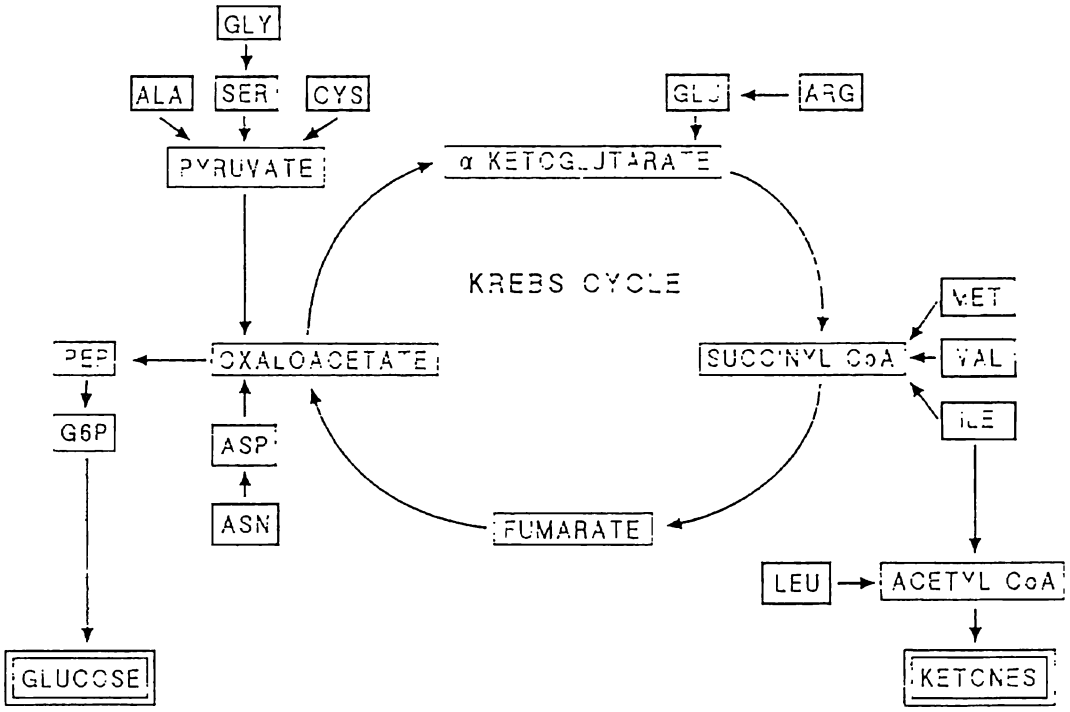

Figure 3. Entry points of carbon skeletons of amino acids into gluconeogenic and ketogenic pathways. Modified diagram from Rocha (134).

The precise mechanism of amino acid-stimulated glucagon secretion is not well documented. Amino acids may enhance glucagon secretion by directly stimulating the islet α cells. Arginine treatment was shown to increase the cytoplasmic Ca^{2+} level of islet

α cells by three fold (80) and an increased cytoplasmic Ca^{2+} level in the α cells is a prerequisite for glucagon secretion (136). On the other hand, amino acids may stimulate glucagon secretion indirectly through neural pathways. It has been suggested that neural receptors for arginine are present in rat liver (157). Binding of arginine to its neural receptors signals the brain stem via the afferent vagal nerve. The efferent vagal nerve is in turn activated and signals the α cells to secrete glucagon. In this study (157), sectioning of the celiac branches of the vagal nerve significantly decreased the arginine-induced glucagon secretion. Therefore, both direct and indirect actions may exist for amino acid stimulation of glucagon release.

Glucose. An elevated plasma glucose concentration suppresses glucagon secretion. Intravenous infusion of glucose lowers plasma glucagon concentration in humans (20, 24, 81), dogs (161) and rats (79). Inhibitory effects of glucose on glucagon secretion also were shown in experiments using the perfused rat pancreas (52).

The cellular mechanism of glucose-induced hypoglucagonemia is uncertain. The effect of glucose on α cell secretion could be direct or indirectly mediated by insulin and somatostatin. Specific glucose receptors or glucose transporters have not been detected on α cells. However, one report (80) showed that the intracellular Ca^{2+} level in isolated α cells was significantly reduced when cells were treated with glucose. Because the release of glucagon requires a rise in intracellular Ca^{2+} concentration (136), glucose may directly suppress α cell secretion by decreasing intracellular Ca^{2+} levels.

Alternatively, glucose-induced hypoglucagonemia may be mediated by insulin and somatostatin. The uptake of glucose into the α cell may be insulin-dependent. Reports (31, 175) showed that the ability of glucose to suppress glucagon secretion in vivo was

diminished in streptozotocin-induced diabetic rats, which lack insulin because their β cells have been destroyed. In vivo studies in humans (34, 71) demonstrated that insulin needs to be present for the inhibitory effect of glucose on α cell secretion. This suggested that paracrine or gap-junction communications between α and β cells may play an important role in the α cell response to glucose stimulation.

Electron micrographs have provided evidence of gap-junctions between α and β cells (163). Extensive studies on intra-islet regulation by Samols (138) indicated that direction of intra-islet microvascular blood flow may determine the extent of intercellular interaction among islets cells. Using the unique microvascular structure of rats islet (23). Samols (138) showed that afferent arterioles entered the islet at the core of islet, so that β cells were the first islet cell type to be perfused. Blood flow then continued to the mantle (i.e., peripheral) area of the islet and reached the α and D cells sequentially. This microvascular $\beta \rightarrow \alpha \rightarrow$D sequence as suggested by Samols (138) implied that elevated plasma glucose was first sensed by β cells. Insulin released upon glucose stimulation could in turn inhibit glucagon secretion from α cells via paracrine communication. Although the microvascular structure of islets reported by Samols (138) was very convincing, it is not clear if this information can be extrapolated to other species.

Hormonal Regulation

Insulin. Insulin inhibits glucagon secretion. In perfused normal rat pancreas, addition of insulin, at euglycemic conditions, inhibited glucagon secretion (31, 175). Continuous infusion of insulin antiserum under euglycemic conditions led to a significant rise in plasma glucagon concentration (97, 138). Exaggerated glucagon secretion is seen in patients with insulin-dependent diabetes mellitus (IDDM) because they have a deficiency

of β cells. Insulin treatment in IDDM patients raised the plasma insulin level and suppressed glucagon secretion (24). In contrast, insulin infusion in obese people with NIDDM had minimum effect on suppressing their plasma glucagon concentration (63), which are results suggesting that the inhibitory action of insulin on α-cell secretion is through paracrine communication. Alternatively, the insulin inhibitory action on the α cells may be a result of direct passage of insulin from β cells into α cells through gap junctions (163).

Somatostatin. The D cells of islets secrete the hormone somatostatin which exerts paracrine effects on neighboring α and β cells. Intravenous injection or infusion of somatostatin suppresses insulin and glucagon secretion in vivo as illustrated by results in humans and sheep infused iv with somatostatin (26, 168). Direct treatment of isolated rat islets with somatostatin also inhibited insulin and glucagon secretion (138). The precise mechanism of somatostatin action on α and β cells is not clear. Presumably, somatostatin decreases the intracellular cAMP level by decreasing mitochondrial and plasma membrane permeability for Ca^{2+} (179). Because secretion of glucagon requires an increase in α-cell content of cytosolic cAMP and Ca^{2+}, somatostatin may inhibit glucagon secretion by decreasing cAMP and Ca^{2+} in α cells.

Growth Hormone. Growth hormone plays a key role in lipid and carbohydrate metabolism. The insulinotropic effect of growth hormone has been well documented in both ruminants (43, 69) and non-ruminants (25, 133, 148). A bolus iv administration of growth hormone increased portal plasma glucagon concentration but not the peripheral plasma level of glucagon in dogs (46, 148). The stimulatory effect of growth hormone

on glucagon secretion also was demonstrated in the perfused rat pancreas (155).

Growth hormone enhances glucagon secretion and the glucagon stimulates hepatic glucose production which leads to an increase in the plasma glucose level. At the same time, growth hormone also directly acts on liver to induce glycogenolysis, and this action of GH further increases the plasma glucose concentration. Prolonged exposure to high doses of growth hormone can lead to permanent diabetes in animals (8, 99).

Sex Steroids. Gestational diabetes is common and potentially can become permanent diabetes in women. Therefore, the effects of female sex steroids on insulin and glucagon secretion are important. Although receptors for progesterone exist in α cells of human islets (39), pregnancy in humans is not associated with abnormal glucagon concentrations (59). Furthermore, the peripheral plasma level of glucagon was unaltered in rats implanted with progesterone or estradiol (9), and co-culture of rat islets with progesterone or estradiol did not affect basal or secretagogue-induced glucagon secretion (82, 118). On the other hand, pregnancy or treatment with female sex steroids invariably leads to β cell hypertrophy, hyperinsulinemia and exaggerated insulin response to glucose in rats (36, 61). This may indicate that insulin, not glucagon, is involved in the impaired glucose homeostasis of pregnancy.

Neural Regulation

Innervation of islet α cells. Pancreatic islets, including the α cells, are innervated by the sympathetic and parasympathetic fibers of the autonomic nervous system (182). Sympathetic fibers from the splanchnic nerve synapse at the coeliac ganglion, and enter the islets together with the vagus nerve as the mixed pancreatic nerve (Figure 4).

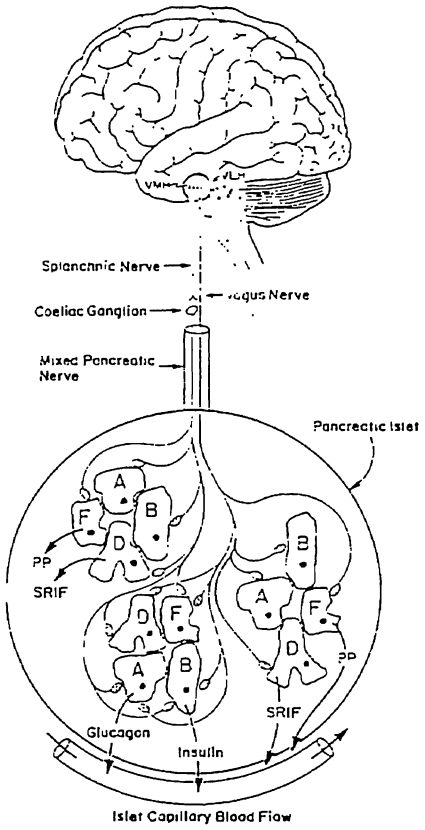

Figure 4. Schematic diagram of neural control of the mammalian pancreatic islet. Not all islet cell are innervated. Considerable species difference exist. Adapted from Hazelwood (65)

Effects of Sympathetic Stimulation. Sympathetic stimulation promotes glucagon release from islet α cells in humans (30) and domestic animals (5, 21, 88). In ruminants, alpha adrenergic, but not beta adrenergic, agonists consistently increased glucagon secretion in goats (121), sheep (120) and adrenalectomized calves (22). However, both alpha$_2$-

adrenergic (57, 73, 137) and beta-adrenergic (52, 75, 142) receptors mediated the sympathetic stimulation of glucagon secretion in nonruminants.

Although species differences and experimental conditions, may explain the conflicting results in ruminant and nonruminants, the effects of insulin on glucagon responses to adrenergic drugs must be considered. Because alpha$_2$-agonists inhibit insulin secretion from β-cells in nonruminants (75, 84, 120, 121), the finding of elevated glucagon concentration in response to alpha$_2$-agonist treatment may simply be due to the attendant decrease in insulin level. A study (142) using isolated rat islet α-cells and β-cells showed that the beta-agonist, isoproterenol, significantly increased cAMP production and glucagon release by the α cell. On the other hand, the alpha$_2$-agonist, clonidine, significantly decreased the cAMP level and insulin secretion by isolated β cells. Therefore, it is likely that sympathetic stimulation of glucagon secretion is mediated by beta-adrenergic receptors whereas adrenergic regulation of insulin secretion is mediated by alpha$_2$-adrenergic receptors.

Sympathetic control of islet hormone secretion is very important under conditions of metabolic and psychological stress and in the defense against hypoglycemic conditions. Activation of the sympathetic input will shut down insulin release and stimulate glucagon secretion, thus increasing hepatic glucose output. The rise in plasma glucose level induces hyperglycemia or reverses the hypoglycemic condition and thus provides immediate substrate for energy generation in muscle and brain to cope with the metabolic or psychological stress.

Effects of parasympathetic stimulation. Parasympathetic stimulation of glucagon secretion was demonstrated in various mammalian species (2, 4, 71, 96, 165). The dorsal trunk

of the vagus nerve innervates islets and is responsible for stimulation of glucagon secretion (83). The mechanism of vagal stimulation of glucagon secretion is controversial. At the neuroeffector junction, the terminal nerve ending releases acetylcholine which increase by interacting with muscarinic receptors in the plasma membrane of α cells glucagon secretion (71, 83). Additionally, peptides released by the vagal terminus may also be responsible for the vagally stimulated rise in glucagon secretion (4).

The presence of parasympathetic tone in control of glucagon secretion is important especially during preabsorptive stage. Because the parasympathetic nervous system stimulates insulin release before dietary glucose is absorbed, the simultaneous release of glucagon may prevent insulin induced hypoglycemia.

GLUCAGON ACTION

Liver is the major target organ for glucagon (163). The most important physiological function of glucagon is to stimulate hepatic glucose production by either glycogenolysis or gluconeogenesis. Glucagon also promotes ketogenesis and ureagenesis in liver and lipolysis in adipose tissue.

Hepatic glycogenolysis

In fasted humans and monogastric animals, liver glycogen is the major supply of hexose units for maintenance of blood glucose level. The breakdown of hepatic glycogen (i.e., glycogenolysis) is stimulated by glucagon.

The glycogenolytic action of glucagon begins with glucagon binding to its receptor on hepatic parachymal cells. The glucagon-receptor complex activates transducer protein,

the G protein, which in turn activates adenylate cyclase (93, 135). Adenylate cyclase catalyses the dephosphorylation of ATP to cAMP. The activated cAMP-dependent protein kinase phosphorylates the enzyme phosphorylase b and converts it to the active form, phosphorylase a. The active enzyme phosphorylase a breaks down glycogen into glucose-1-phosphate (114). Phosphorylase a is the rate-limiting enzyme in the glycogenolytic pathway. The glucose 1-phosphate formed is converted to glucose 6-phosphate by the enzyme phosphoglucomutase. The final step in hepatic glycogenolysis is catalyzed by glucose 6-phosphatase, which acts by removing the phosphate group from glucose 6-phosphate to allow free glucose to diffuse from hepatocytes into the blood for circulation.

The hepatic content of glycogen is relatively low in ruminants as compared with nonruminants. Glucose uptake by the liver is deficient in ruminants due to their low activity of hepatic glucokinase (17). Glucagon stimulation of hepatic glucose output in domestic ruminants is due mainly to glucagon stimulation of hepatic gluconeogenesis and, to a lesser extent, hepatic glycogenolysis.

Hepatic gluconeogenesis

In nonruminants, including humans, gluconeogenesis is physiologically activated only when hepatic glycogen is depleted and exogenous glucose is not available, such as in prolonged fasting. In ruminants, however, dietary carbohydrates are fermentated to volatile fatty acids and little glucose is absorbed through the gastrointestinal tract (18). Unlike nonruminants, the maintenance of euglycemia in ruminants relies on continuous hepatic gluconeogenesis. Thus hepatic gluconeogenesis is greater in the fed than fasted ruminant. The biochemical mechanisms for endocrine control of hepatic gluconeogenesis, however, are similar in ruminants and nonruminants.

Hepatic gluconeogenesis is the process of converting noncarbohydrate precursors, such as amino acids, glycerol and propionate, to glucose in liver. Amino acids enter the gluconeogenic pathway as pyruvate or via intermediates in the TCA cycle. Transaminases are enzymes that transaminate amino acids to pyruvate or to oxaloacetate in the hepatocyte cytosol. Pyruvate enters the mitochondrion and is converted to oxaloacetate by pyruvate carboxylase. Decarboxylation of oxaloacetate to phosphoenolpyruvate (PEP) by PEP carboxykinase (PEPCK) is one of the rate-limiting steps in the gluconeogenic pathway. Glucagon promotes gluconeogenesis by stimulating the activity of PEPCK. Phosphoenolpyruvate is converted to fructose 1,6-bisphosphate. The dephosphorylation of fructose 1,6-bisphosphate to fructose 6-phosphate is catalyzed by fructose 1,6-bisphosphatase and this step is another important rate limiting step of gluconeogenic pathway. Glucagon enhances gluconeogenesis by decreasing the amount of fructose 2,6-bisphosphate, which functions as an allosteric inhibitor of fructose 1,6-bisphosphatase. In liver, conversion of glucose 6-phosphate into glucose requires the enzyme glucose 6-phosphatase. The removal of the phosphate allows free glucose to transverse the liver cell membrane and enter blood.

Free fatty acids and glycerol are products of lipolysis, but only glycerol is gluconeogenic. Glycerol enters the gluconeogenic pathway at the triose phosphate stage via conversion by glycerol kinase and glycerol-3-phosphate dehydrogenase.

Propionate is a major source of glucose in fed ruminants. In liver cell mitochondria, propionate is converted to succinyl-CoA which enters the gluconeogenic pathway. The gluconeogenic precursors are similar in fasted ruminants and nonruminants because propionate is only present in fed ruminants.

Hepatic ketogenesis

Ketogenesis occurs under physiological conditions as well as in pathological situations such as in diabetic ketoacidosis, pregnancy ketosis, and lactational ketosis in high-yielding dairy cows. Ketone bodies (acetoacetate, β-hydroxybutyrate and acetone) are products of hepatic ketogenesis. Ketone bodies can be used as an energy source by all tissues including certain parts of the brain.

Glucagon stimulates and insulin inhibits ketogenesis. In the fasted state, decreased plasma insulin levels and elevated plasma glucagon promote adipocyte lipolysis, which in turn increases the plasma concentration of free fatty acids (FFA). Cellular uptake of plasma FFA is positively related to plasma FFA concentration; therefore, the amount of fatty acid in the hepatocyte cytosol rises in response to lipolysis. Glucagon stimulates mitochondrial uptake of activated fatty acid (fatty acyl-CoA) by inducing the activity of fatty acid transporting enzyme known as hepatic carnitine acyl transferase that is located in the inner mitochondrial membrane. Fatty acyl-CoA is subsequently oxidized (β oxidation) to acetoacetyl-CoA inside the mitochondrion. The acetoacetyl CoA can either be directly deacylated to acetoacetate by deacylase enzyme, or acetylated to 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) which in turn can be converted to acetoacetate by HMG-CoA lyase. Acetoacetate is one of the ketone bodies; it spontaneously decarboxylates to form acetone, or it can be enzymatically reduced to β-hydroxybutylate by D(-)-3-hydroxybutyrate dehydrogenase (114, 158). The rate-limiting step in hepatic ketogenesis is the rate of lipolysis in adipose tissue because this ultimately determines the hepatic cytosol content of fatty acids (117)

Glucagon effects on protein metabolism

Glucagon increases protein catabolism (58) in muscle to provide amino acids for gluconeogenesis. Glucagon-induced gluconeogenesis produces an excessive amount of amines which enter the urea cycle. Glucagon directly stimulates the activities of all enzymes of the urea cycle and thus promotes urea synthesis and prevents accumulation of toxic ammonia (3).

Glucagon as a counter-regulatory hormone

Insulin is a major regulatory hormone of glucose metabolism. Glucagon, growth hormone and cortisol physiologically counter the effects of insulin and thus are referred to as counter-regulatory hormones.

Insulin inhibits but glucagon stimulates glycogenolysis, lipolysis and ketogenesis. Because of these opposing actions of insulin and glucagon, the molar insulin:glucagon ratio (I:G) perfusing hepatic tissue determines the balance of hepatic metabolism. Hepatic glucose output is determined by the I:G ratio. In the fasted state, when blood glucose level tends to decline, I:G ratio decreases and glycogenolysis and gluconeogenesis are stimulated. The reverse occurs during the fed state. The decrease in I:G ratio during the fasted state is due mainly to a decrease in insulin secretion although a slight increase in glucagon concentration also occurs. Similarly, the increase in I:G ratio in the fed state is mainly due to an increase in insulin level instead of a major decrease in glucagon secretion.

Glucagon actions on cardiovascular system

Cardiac muscle contraction is strenghtened after intravenous injection of glucagon,

or after adding glucagon to isolated or cultured heart cells. (51, 76). However, glucagon induces vasorelaxation in dog blood vessels in vitro (123). The inotropic effect of glucagon in the heart is mediated by activation of adenylate cyclase (107) which subsequently leads to a rise in the cAMP level of the target cell (112). Although high concentrations of glucagon have inotropic effects in cardiac tissue, the picomolar concentrations of glucagon normally present in peripheral blood are too low to cause any change in the cardiovascular system under physiological conditions (126).

Glucagon effects on electrolyte balance

Glucagon stimulates renal reabsorption of Na^+, K^+, Cl^-, Mg^{2+} and Ca^{2+} (13, 14, 40), but it inhibits bicarbonate reabsorption (55, 106) in the thick ascending limb of Henle's loop in rats. In the small intestine, glucagon caused Cl^- secretion in mice in vitro (85) and in humans in vivo (68). The glucagon-induced outward movement of Cl^- from enterocytes causes excessive intestinal fluid secretion (92). An elevated glucagon levels stimulate an excessive intestinal fluid secretion which overloads the absorptive ability of the colon, and results in diarrhea as commonly observed in terminal-stages victims of famine and severe malnutrition.

Glucagon receptors

Glucagon receptors are mainly located on membranes of hepatic parachymal cells, but they are also found in adipocytes, brain tissues (145) and pancreatic islet cells (1). Hepatic glucagon receptors have a molecular weight of approximately 190 kDa (65). The 6 amino acids at the N-terminus (His-Ser-Gln-Gly-Thr-Phe) of glucagon are required for specific binding to its receptor. Removal of histamine from the N-terminal region of

glucagon prevents its binding to receptor and thus terminates all biological activities of glucagon (45). Amino acids at region 10-25 of the receptor form a helical structure when the receptor is occupied by glucagon and this conformational change in the occupied receptor may maintain a glucagon-receptor complex long enough to initiate a biological action. Once formed, the glucagon-receptor complex undergoes receptor-dependent endocytosis (10). The ligand, glucagon, is degraded in endosomes (11) and the receptors, devoid of glucagon, remain intact to be either recycled to the plasma membrane or degraded intracellularly (10).

GLUCAGON KINETICS

Glucagon secretion

Glucagon produced by pancreatic α cells is secreted into the portal vein which perfuses the liver, the major target tissue for glucagon. Due to the unique direct blood flow from pancreas to liver, the majority of the secreted glucagon is degraded by the liver before it enters the peripheral circulation. Therefore, plasma concentrations of glucagon are much higher in portal than in peripheral blood. Conclusions regarding the pancreatic secretory rates of glucagon based solely on the fasting plasma glucagon concentrations (37, 49) may be misleading in part because they ignore the portal-peripheral blood gradient of glucagon concentration. In addition, direct measurement of plasma glucagon by radioimmunoassay (RIA) is fraught with problems due to interference in the RIA by cross-reacting large molecular weight plasma proteins (discussed in later section) which lead to an overestimation of the true level of plasma glucagon.

Glucagon secretion rate has been measured indirectly in several species by kinetic

analysis of the plasma disappearance of iv injected glucagon. Estimates of glucagon secretion rate are similar in fasted humans, cows and sheep (Table 1). Direct measurement of glucagon secretion in vivo has been done in fed sheep.

Table 1. *Estimated secretion rate of glucagon in different species*

Species	Physiological condition	Secretion Rate (pmol · min^{-1} · kg^{-1})	Half Life min	References
Humans	fasted	0.37	-	49
Humans	fasted	-	4.8	7
Dairy cows	fasted	0.32	7.7	37
Sheep	fasted	0.31	23	26
Sheep	fed	0.61	-	27
Rats	fed	-	2.5	15
Dogs	fasted, anesthetized	-	5.5	77
Pigs	Sham Operated	1.60	57	167

Glucagon secretion rate and biological half life were estimated by kinetic analysis of glucagon disappearance from plasma, except for sheep (26) and Dog (77) where pancreatic secretion rate of glucagon was calculated as plasma concentration difference between the portal and caudal aorta blood vessels multiplied by portal plasma flow. Glucagon concentrations used in kinetic analysis were from the direct measurement of plasma immunoreactive glucagon by RIA.

Glucagon degradation

Liver is the major site for glucagon degradation in sheep (27), rats (116), dogs (77) and humans (124). Some glucagon and majority of the proglucagon gene products are degraded by the kidney (124). Proteolytic enzymes in plasma also degrade large amounts of glucagon.

Secreted glucagon enters the liver via the portal vein. Upon interaction with the glucagon receptors in hepatocytes, the glucagon-receptor complex undergoes receptor-mediated endocytosis (62). After dissociation from the receptor in endosomes or lysosomes, the free glucagon is degraded (11).

The biological half-life ($t_{\frac{1}{2}}$) measures the whole-body metabolism of hormone and it is the time required for the circulating concentration of a hormone to decrease by 50%. The $t_{\frac{1}{2}}$ of glucagon is quite variable among species (see Table 1), ranging from 2.5 min in rats (15) to 56 min in pigs (167). Certain physiological states such as obesity in sheep (unpublished data), high protein diet in rats (15) and lactation in cows (37) did not change the $t_{\frac{1}{2}}$ of glucagon. However, specific physiological disorders such as liver cirrhosis, kidney failure (143) and diabetes mellitus (7) significantly prolonged the $t_{\frac{1}{2}}$ of glucagon.

MOLECULAR SPECIES OF IMMUNOREACTIVE GLUCAGON

Glucagon specific antisera

As discussed previously, multiple peptides originate from proglucagon gene expression in the pancreas and intestine of humans. Antisera raised against glucagon may cross-react with peptides derived from proglucagon because these peptides contain some homologous sequences to glucagon. Therefore, radioimmunoassays for glucagon may measure glucagon alone or glucagon plus other immunoreactive components of the proglucagon gene.

Glucagon specific antisera are classified into two major types based on their selectivity towards the N-terminus or C-terminus of glucagon. N-terminal antisera recognize glucagon itself and other proglucagon-derived peptides as well as some large immunoreactive plasma proteins. The C-terminal antisera, however, only measure glucagon and the large immunoreactive plasma proteins (70).

Accurate measurement of plasma glucagon

Accurate measurement of plasma glucagon requires a glucagon specific C-terminal antisera. Many laboratories have produced C-terminal antisera, the most commonly used and best recognized antiserum is Unger's 30K C-terminal glucagon specific antiserum raised by R.H. Unger in 1959 (159). Although C-terminal antisera do not cross-react with glicentin and GLP-1 (160), but they do recognize the large immunoreactive plasma proteins (166, 177). The identity of such large immunoreactive plasma proteins is not known. The immunoreactive proteins are composed of at least two proteins of different molecular weight in humans (181). Chromatographs of human plasma showed that the larger and the smaller sized immunoreactive plasma protein eluted at position corresponding to the gamma globulin and bovine serum albumin markers, respectively. Removal of IgG from plasma by staphylococcal protein A adsorption also removed the larger immunoreactive protein from human plasma (175). A sudden increase in total IRG level in sham-operated pigs (167) and burned patients (109) also supports the idea that the larger immunoreactive plasma protein is IgG.

Direct measurement of plasma glucagon by RIA using C-terminal antiserum will overestimate its true concentration. Organic solvents such as ethanol (50) and acetone (169) has been validated for use in precipitating the large immunoreactive plasma proteins and leaving the majority of the glucagon intact in the supernatant. In this thesis, polyethylene glycol (PEG) was validated as an efficient agent to precipitate the large immunoreactive plasma proteins. Therefore, accurate measurement of plasma glucagon concentration can be achieved by RIA measurement of ethanol, acetone or PEG treated plasma using C-terminal glucagon specific antiserum.

GLUCAGON IN OBESITY

Molecular profiles of basal plasma IRG are similar in normal humans (78, 150) and in dogs (77). Plasma total IRG, as measured by C-terminal antisera, was composed of a glucagon peak and a large immunoreactive protein peak with molecular weight of 60 kDa or larger. Chromatograph of normal human plasma on a Sephadex G200 (2.6 x 100 cm) column provided results to demonstrate that the large immunoreactive proteins were composed of at least two distinct proteins of molecular weight 160 kDa and 68 kDa (176). The large immunoreactive protein peak was found not affected by obesity in humans. However, its concentration is variable among individual regardless of body condition (150).

The effects of obesity on fasting levels of basal glucagon in plasma are not clearly established. Studies based on plasma levels of immunoreactive glucagon (IRG) found that obese humans and animals were either hypoglucagonemic (139, 140), hyperglucagonemic (79, 102, 150) or euglucagonemic (81, 108, 129). Because total IRG is comprised of both the large immunoreactive plasma proteins and glucagon, these conflicting reports may simply be due to differences in the degree of interference by the large immunoreactive proteins in the RIA for glucagon. Starke (150) used chromatography to separate the large immunoreactive proteins from glucagon and Pouilliot (127) used polyethylene glycol to precipitate the large immunoreactive proteins from plasma before the samples were subjected to RIA. Both of these (127, 150) studies unequivocally showed that obese humans were hyperglucagonemic.

The effects of obesity on α cell responsiveness to glucagon secretagogues also are not clearly established. Exaggerated (79, 81) or normal (73, 108) plasma glucagon responses to arginine or alanine stimulation have been reported in obese as compared

with lean humans and animals. The cellular and molecular basis for the fasting hyperglucagonemia and exaggerated α cell responsiveness to glucagon secretagogues is not known but it may be related to the presence of insulin resistance in α cells. Insulin normally inhibits glucagon secretion (31, 175). Plasma glucagon concentration was decreased less in obese than lean humans when each was exposed to hyperinsulinemia and hyperglycemia (24) and to hyperglycemia during euglycemic condition (63).

Chapter III

QUANTIFICATION OF PLASMA GLUCAGON

INTRODUCTION

The proglucagon gene is expressed in alpha cells of the pancreas and intestine in several animal species (41, 110, 119). At least five-to-six bioactive peptides can be derived from proglucagon mRNA (70, 78). Glucagon is the major secretory product of the proglucagon gene in pancreatic alpha cells, whereas glicentin is the major secretory product in intestinal cells. Pancreatic glucagon is responsible for the endocrine regulation of hepatic glycogenolysis and gluconeogenesis.

Accurate measurement by radioimmunoassay (RIA) of pancreatic glucagon (3,500 daltons) can be problematic because of the presence in plasma of large molecular weight immunoreactive proteins and multiple products of the proglucagon gene that crossreact with glucagon antisera (166, 176, 170). Antisera directed against the carboxyl-terminus of glucagon specifically recognize pancreatic glucagon but not other products of the proglucagon gene such as glicentin, GLP-1, GLP-2 or the intact proglucagon polypeptide (160). However, these so called C-terminus specific antisera still crossreact with large molecular weight plasma proteins that express glucagon-like immunoreactivity (149). RIA systems that use C-terminus specific antisera therefore measure immunoreactive glucagon (IRG) concentrations which are composed of glucagon and the large immunoreactive protein components. The large immunoreactive proteins can be removed by treating

plasma with ethanol or acetone to precipitate out the large immunoreactive component of IRG (50, 169, 173). Von Schenck used gel chromatography to show that acetone extraction of plasma removed approximately 90% of its large immunoreactive protein content while allowing approximately 80% recovery of the pancreatic glucagon.

Extracting plasma with ethanol or acetone requires an evaporative step to remove the solvent and a sample reconstitution step before the sample is ready for assay. Each of these steps increases the potential for analytical error and at the least should increase the imprecision of the RIA. Physically separating the large immunoreactive proteins and glucagon by passing plasma through a molecular sieving membrane should provide optimum ease of separation and minimum dilution of the glucagon concentration. However, we found this approach not be feasible because preliminary experiments showed that approximately 90 percent of the plasma glucagon content was retained by the passivated and non-passivated membrane. Therefore, the use of polyethylene glycol (PEG) as an alternative means of removing the large immunoreactive proteins was considered.

PEG should be suitable for selectively precipitating the cross-reacting large molecular weight proteins because PEG sterically excludes large molecular weight proteins from their solubility limit (74) and has minimum effect on small polypeptides (38, 116). A PEG-extraction method should provide a simple and consistent means to extract glucagon because PEG extraction does not require solvent evaporation and reconstitution steps. PEG-extraction method has been used to measure glucagon in humans (127), but a validation of this method has not been reported.

The quality of tracer glucagon used in the RIA is also a limiting factor in accurate quantification of plasma glucagon. Different iodination procedures for glucagon have

been studied (144, 172, 181). The quality of iodinated glucagon usually has been determined by the percentage of tracer that can be precipitated with trichloroacetic acid (TCA) or by incubation with excessive amounts of glucagon antisera. We have found these two approaches to be inconsistent in identifying quality [^{125}I]glucagon.

The objectives of this study were to validate a PEG-extraction procedure for accurate quantification of pancreatic glucagon by RIA and to identify a strategy for testing and selecting quality glucagon tracer prepared by three different iodination procedures.

MATERIALS AND METHODS

In this paper, the term immunoreactive glucagon (IRG) will refer to measurement by RIA of plasma glucagon immunoreactivity which includes that due to glucagon (3,500 Da) and large ($\geq$ 61 kDa) immunoreactive proteins; the term glucagon refers to measurement by RIA of glucagon (3,500 Da) concentrations in plasma.

Glucagon Radioimmunoassay

A C-terminal specific polyclonal rabbit anti-glucagon serum previously validated for measuring plasma IRG and glucagon in sheep by RIA (101) was used in this experiment. Radioimmunoassays using this or Unger's 30K antisera provide similar measurements of glucagon and IRG in sheep and canine plasma. Crystallized bovine glucagon (Lilly Research Laboratories, Indianapolis, IN) was used as standard. As described later, the chloramine-T method (pH 7.4) was used routinely to prepare [^{125}I]glucagon. Assay buffer (pH 8.7) contained 0.05 M Trisma base, 0.01 M EDTA, 15 mM sodium azide, 16 mM benzamidine hydrochloride, 0.03 M sodium chloride and 0.5% bovine serum albumin. All reagents were purchased from Sigma Company (St Louis, MO) unless otherwise

stated. Assay conditions were as follows. Two hundred microliters of glucagon standard (12.5, 25, 50, 100, 200 and 400 pg/ml) or plasma samples were coincubated in glass tubes with 0.5 ml glucagon antisera diluted 1:12,000 in assay buffer for 18 - 24 h at 4°C. Approximately 20,000 CPM of [^{125}I]glucagon were added (0.1 ml) and tubes incubated further for 18 - 24 h at 4°C. Goat anti-rabbit gamma globulin (Calbiochem, San Diego, CA) diluted 1:250 (id) in assay buffer was added (0.2 ml) and the incubation continued for an additional 72 h at 4°C. The assay was terminated by the addition of 3.8 ml cold (4°C) rinse buffer (pH 8.7) which contained 0.05 M Trisma base, 2 mM EDTA and 15 mM sodium azide. The precipitated antibody complexes were pelleted by centrifuging at 1,500 x g for 45 min at 4°C. After decanting the supernatant, radioactivity in the pellet was quantified by counting in a gamma counter.

Preliminary data showed that the presence of residual PEG in PEG-extracted plasma led to a 4-fold overestimation of glucagon concentration by RIA (data not shown). Presumably, the residual PEG altered the binding properties of the first and second antibodies. As a result, antibody-bound and free [^{125}I]glucagon were not reliably separated by the double antibody procedure. A modified RIA procedure, therefore, was developed for measuring glucagon in PEG-extracted plasma. In the modified RIA, PEG was used in place of goat anti-rabbit IgG to separate antibody-bound and free [^{125}I]glucagon. Assays using the PEG-separation method were terminated 48 h after the addition of [^{125}I]glucagon by adding 0.2 ml of pooled sheep plasma and then 1.5 ml of PEG (8000 mol wt; 18%) in cold rinse buffer to the assay tubes. Assay tubes were vortexed, centrifuged at 1,500 x g for 45 min at 4°C and the resulting supernatant was aspirated before the precipitates were counted for radioactivity.

PEG Extraction Method

Procedure for the PEG removal of large immunoreactive proteins from sheep plasma was based on that used for precipitation of antibody-bound insulin (115). Briefly, equal volumes of plasma sample and 25% PEG (8,000 mol wt) in rinse buffer were vortexed for one minute then centrifuged at 1,500 x g for 45 min at 4°C. The supernatant (i.e., PEG-extracted plasma) was collected for use in the RIA.

Efficacy of PEG Extraction in Removing the Large Immunoreactive Plasma Proteins

The efficacy of the PEG-extraction procedure in removing large immunoreactive proteins from plasma was studied in several ways. First, five milliliters of unextracted plasma and 5 ml of PEG-extracted plasma were chromatograhed separately on a Sephadex G50-50 gel column (1.5 x 80 cm). The column was calibrated with molecular markers, namely blue dextran and vitamin B_{12}. RIA buffer was used as the elution buffer. Fractions (2 ml) collected were assayed for IRG as described above except that 0.8 ml standard or fraction eluent were assayed and PEG was used to separate antibody-bound and free [^{125}I]glucagon in the RIA.

Surprisingly, it was found that the large immunoreactive proteins were barely or not detectable in the chromatographs of unextracted plasma when PEG was used in the RIA to separate antibody-bound and free [^{125}I]glucagon (Figure 5).

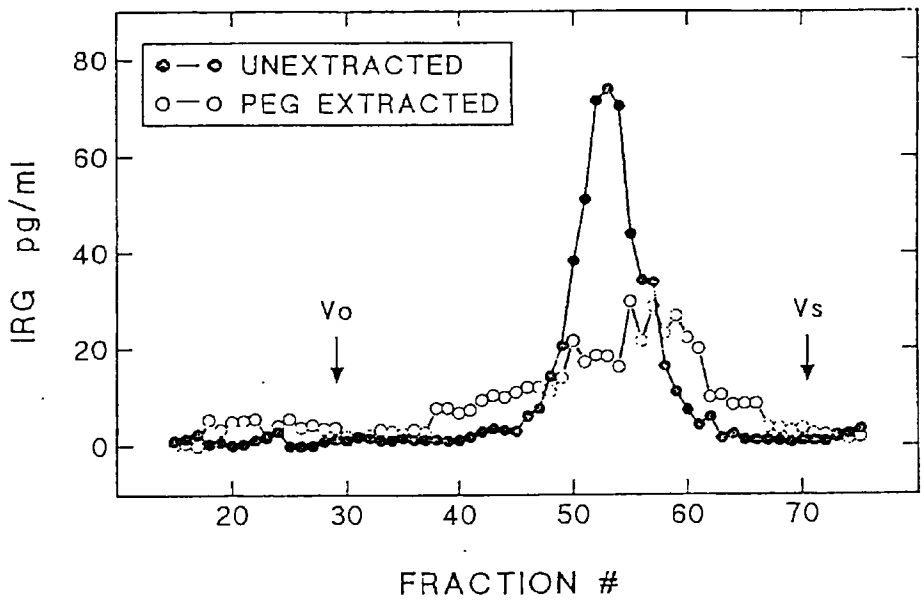

Figure 5. Molecular profile of IRG in plasma before and after PEG extraction. Five milliliters of sheep plasma were chromatographed on a Sephadex G50-50 (1.5 x 80 cm) column. Void volume (Vo) and salt peak (Vs) of the column were marked by blue dextran and vitamin B_{12}, respectively. Fractions collected were assayed for their IRG content by RIA using PEG to separate bound and free [^{125}I]glucagon. Note the presence of a glucagon peak (fraction 46 to 60) but the absence of the large immunoreactive peak in fractions eluting immediately after the Vo).

Chromatographs of unextracted sheep plasma in previous work routinely had been assayed under similar conditions except that goat anti-rabbit IgG was used to separate antibody-bound and free [^{125}I]glucagon. A glucagon peak and two peaks due to the large immunoreactive proteins typically were observed under those conditions. PEG was used here because the double antibody procedure produced artifactual concentrations of IRG when PEG-extracted plasma was assayed by RIA employing second antibody to separate the antibody-bound and free [^{125}I]glucagon. Presumably, the double antibody and PEG separation methods in RIA provided different measurements of plasma IRG. This result led us to rechromatograph unextracted sheep plasma on a larger column containing high resolution gel (Sephacryl 100HR, 2.6 x 100 cm) in order to resolve this discrepancy. An aliquot (0.8 ml) of each fraction was measured for IRG content by RIA either using

double antibody method or PEG method to separate antibody-bound and free [^{125}I]glucagon.

Additionally, the results for the chromatograph of the PEG-extracted plasma in Figure 5 were inconclusive because the residual PEG in the extracted plasma affected adversely the flow properties of the gel. Changing the column eluent to include 3% PEG did not improve the quality of the chromatographic results. Thus, this first approach to test if PEG removed large molecular weight IRG was inconclusive.

Because of the above problems, an alternative experiment was designed to test the efficacy of PEG in removing the large immunoreactive protein components of plasma (Figure 6). Dextran-coated charcoal was used as a means to remove glucagon (3500 Da) from plasma. If charcoal-absorbed plasma is glucagon-free, then the IRG content of charcoal-absorbed plasma would represent only the large immunoreactive proteins. Therefore, the IRG concentration of charcoal-absorbed plasma should become zero when subjected to PEG extraction if indeed the PEG extraction procedure removes all large immunoreactive proteins.

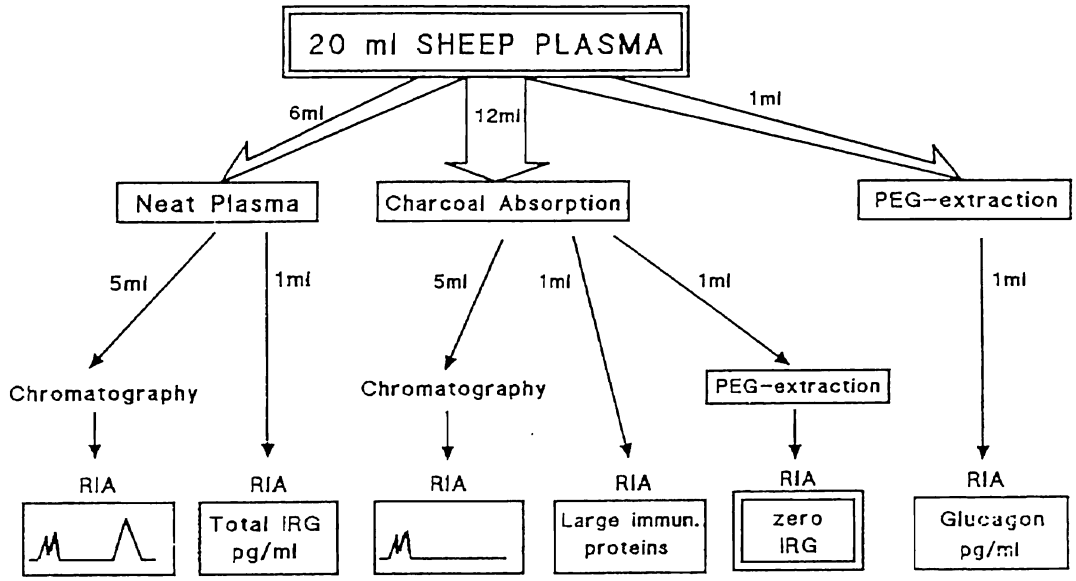

Figure 6. Experimental protocol for testing the efficacy of PEG-extraction in removing large immunoreactive proteins from plasma. The chromatographic profile of neat plasma assayed by RIA using a double-antibody separation procedure should contain 3 peaks, namely the large immunoreactive protein peaks and the later eluting glucagon peak.

A pool of sheep plasma was used to study the efficacy of PEG in removing the large immunoreactive proteins. Six milliliters of untreated sheep plasma were used to determine the plasma IRG content and the chromatographic or molecular profile of IRG. The molecular profile of plasma IRG was obtained by chromatographing 5 ml of untreated plasma on a Sephadex G50-50 (1.5 x 80 cm) column and determining the IRG content of the fractions by RIA. Plasma total IRG concentration was determined by assaying directly an aliquot of the neat plasma.

Twelve milliliters of the same plasma pool were subjected to charcoal absorption to

produce a sample that contained only large immunoreactive proteins (i.e., glucagon-free plasma). The charcoal absorption procedure was designed as follows. Dextran-coated charcoal was prepared by mixing equal volumes of 32% activated charcoal (untreated powder, 100-400 mesh) and 16% dextran (~ 73,000 daltons) in rinse buffer. The plasma was divided into six aliquots (2 ml), each of which were mixed with 0.25 ml of the dextran-charcoal mixture. The plasma-dextran-charcoal mixtures were incubated at room temperature for ten minutes, then centrifuged at 2,000 x g for 15 min. Supernatants from the six aliquots were pooled and filtered through a 0.45 μm membrane (Millipore, Bedford, MA) to remove any residual charcoal powder.

In order to confirm the ability of charcoal to absorb glucagon without affecting the large immunoreactive proteins, 5 ml of the charcoal-absorbed plasma were chromatographed on the Sephadex G50-50 (1.5 x 80 cm) column and column fractions were assayed for IRG. One milliliter of the charcoal-absorbed plasma was assayed directly to determine its IRG content, which should represent the concentration of only the large immunoreactive proteins in plasma. Another aliquot (1 ml) of the charcoal-absorbed plasma was extracted with PEG. If the PEG extraction method completely removes all the large immunoreactive proteins from plasma, then the IRG concentration in the PEG-extracted and charcoal-absorbed plasma should be zero.

Finally, the IRG content in PEG-extracted plasma was measured in order to check the additivity of each procedure. Summation of the IRG concentration measured in PEG-extracted plasma and charcoal-absorbed plasma should equal the total IRG content of the unextracted plasma as measured by RIA.

Goat anti-rabbit IgG was used to separate antibody-bound and free [^{125}I]glucagon in RIA using neat plasma, charcoal-absorbed plasma and fractions from chromatography of

neat plasma or charcoal-absorbed plasma. For PEG-extracted plasma samples, IRG was measured by RIA using PEG to separate antibody-bound and free [^{125}I]glucagon.

Validation of RIA for Measuring Glucagon in PEG-extracted Plasma

Validation of the glucagon RIA using the double antibody method was reported elsewhere (101). Validation of the RIA for quantifying glucagon in PEG-extracted plasma was done as follows.

Accuracy is defined as the extent to which measurements of glucagon agree with the exact amount that is present in a sample. Accuracy of the RIA measurement of glucagon in PEG-extracted plasma was evaluated by adding 25 μl of bovine glucagon to aliquots (975 μl) of sheep plasma to produce final added glucagon concentrations of 0, 25, 50, 100, 150 and 200 pg/ml. The glucagon-spiked plasma samples were then extracted with PEG as described above. The amount of glucagon recovered in the glucagon-spiked plasma after its extracting with PEG was quantified by RIA using PEG to separate antibody-bound and free [^{125}I]glucagon. Recovery of glucagon in the PEG-extraction procedure also was examined as follows. Approximately 50,000 CPM of [^{125}I]glucagon were mixed with 800 μl of untreated sheep plasma. Subsequently, 150 μl of 35% PEG in rinse buffer were added, and the mixture vortexed and centrifuged at 1,500 x g for 45 min at 4 °C. The supernatants were retrieved and counted for radioactivity.

Precision is defined as the extent to which a series of measurements on a sample agree with the mean concentration. Intra-assay precision of the RIA was determined by calculating the coefficient of variation (CV) for 5 repeat measurements each of PEG-extracted sheep plasma pools that contained low (~70 pg/ml), medium (~90 pg/ml), and high (~140 pg/ml) concentrations of glucagon; interassay CV was calculated by

running five different assays on 3 plasma pools that had glucagon concentrations of approximately 50 pg/ml, 90 pg/ml, and 200 pg/ml. Consistency of the PEG-extraction procedures per se was determined by extracting ten aliquots of three plasma pools, and measuring the glucagon content in the extracted plasma by RIA using PEG to separate the antibody-bound and free [^{125}I]glucagon.

Specificity is defined as the degree to which the assay measures what it is intended to measure. Crossreactivity of the antisera and tests of parallelism indicate the specificity of the system. In this experiment, specificity of the assay system was evaluated by comparing the degree of parallelism between binding inhibition curves for glucagon standards with those for serial dilutions of PEG-extracted plasma pools and glucagon standards run in assay buffer containing PEG at an equivalent amount as found in extracted plasma. As mentioned before, some residual PEG is present in PEG-extracted plasma. Whether or not this residual PEG affects assay performance was determined by evaluating the parallelism between standard curves run in the presence and absence of PEG.

Sensitivity is defined as the smallest amount of glucagon that can be distinguished from no glucagon. Assay sensitivity was determined by calculating the hormone concentration equal to the lower 95% confidence interval of the percentage of binding for B_0 assay tubes of five different assays.

Immunological Properties of the Large Immunoreactive Proteins

Because of the interesting observations regarding the detection of the two large immunoreactive proteins (see Figure 7), this study briefly investigated the immunological characteristics of these proteins as regards their ability to bind [^{125}I]glucagon or to

displace antibody-bound glucagon. This was done by three different approaches.

First, the possible presence of a glucagon-binding protein (GBP) in sheep plasma was determined by comparing the chromatographs of [^{125}I]glucagon (approximately 20,000 CPM) previously incubated in RIA buffer (1 ml) alone or buffer (0.8 ml) plus 0.2 ml of pooled sheep plasma. Samples were chromatographed on a Sephadex G25 (1 x 25 cm) and 1 ml fraction collected. The volume ratio of plasma:buffer:[^{125}I]glucagon in the preincubation mixture was identical to that in the incubation conditions of the RIA. If a binding-protein for glucagon exists, then the chromatographic profile of [^{125}I]glucagon peak should be resolved into two peaks of free [^{125}I]glucagon and a protein-bound [^{125}I]glucagon peak when [^{125}I]glucagon was preincubated with plasma before chromatography. If this test for glucagon-binding protein was positive, then 32 ng unlabelled glucagon would be coincubated with the plasma-[^{125}I]glucagon mixture and the chromatography repeated. It was expected that the size of the protein-bound [^{125}I]glucagon peak would diminish in the presence of unlabelled glucagon because the unlabelled glucagon would displace [^{125}I]glucagon from the protein-bound-[^{125}I]glucagon complex.

The second test determined if the $\geq$145 kDa and 61 kDa protein functioned immunologically by binding [^{125}I]glucagon or by displacing antibody-bound [^{125}I]glucagon. Sheep plasma was chromatographed on a Sephacryl 100 HR (2.6 x 100 cm) column as described earlier and the fraction containing the greatest immunoreactive content of the $\geq$145 kDa and 61 kDa proteins were each incubated overnight with approximately 40,000 CPM [^{125}I]glucagon in 200μl RIA buffer. The mixtures then were chromatographed on a Sephacryl 100 HR (1 x 25 cm) column. This was done to show if either of the large immunoreactive proteins were capable of binding [^{125}I]glucagon. In

addition, [^{125}I]glucagon was coincubated overnight with glucagon antisera (1:12K) alone, antisera plus $\geq$145 kDa protein, and antisera plus 61 kDa protein, respectively. The mixtures were chromatographed on the same Sephacryl 100 HR (2.6 x 100 cm) column and fractions (1 ml) collected were counted for radioactivity. This was done to determine the glucagon-binding or glucagon-like antigenicity of the large immunoreactive proteins. The chromatogram of [^{125}I]glucagon that had been previously incubated with glucagon antisera should contain a free [^{125}I]glucagon peak and an antibody bound-[^{125}I]glucagon peak. If the large immunoreactive proteins bind [^{125}I]glucagon then the height of antibody bound peak would be increased, whereas the height of antibody bound peak would be decreased if either of the large immunoreactive proteins expressed similar antigenic determinant as glucagon standard. This test relied on the fact that the antibody-bound [^{125}I]glucagon eluted at the void volume.

The third test was based on the suggestion by Von Schenk (170) that IgG may be part of the large immunoreactive protein complex. This was to demonstrate that sheep IgG is measured by glucagon RIA system as IRG. An equivalent amount (13.5 mg/ml) of ovine IgG as normally found in plasma (54) was dissolved in RIA buffer which contined 5 ng of glucagon. Fifteen milliliters of the IgG -glucagon solution were chromatographed on a Sephacryl 100 HR (2.6 x 100 cm) column. Fractions (5 ml) collected were assayed for glucagon by double antibody RIA.

Glucagon Iodination

Glucagon (Lilly Research Laboratories) for iodination was dissolved in 0.1 M acetic acid and aliquots (1 μg/μl) were stored at -20°C. Iodination of glucagon was accomplished by three methods, namely chloramine T at pH 7.4, chloramine T at pH 10,

and lactoperoxidase at pH 10. For all iodination methods, 5 μg (5 μl) of glucagon was reacted with 0.5 mCi (5 μl) NaI^{125} in a 1 ml polypropyrene microcentrifuge tube that contained 20 μl of iodination buffer.

Chloramine T iodination was done according to the procedure of Shima et al (144). Briefly, glucagon was added to the reaction vial containing 20 μl phosphate (0.2 M, pH 7.4) or Tris (0.015 M, pH 10) iodination buffer. Five microliters of NaI^{125} (Amersham, Arlington Heights, IL), 15 μl of dimethyl sulfoxide (DMSO) and 70 μg chloramine T in 20 μl iodination buffer were added sequentially to the reaction vial. Reaction was allowed for 15 sec before the sequential addition of 12 mg sodium metabisulfide in 50 μl iodination buffer and 25 μl of 25% bovine serum albumin (BSA) in dH_2O. The reaction mixture was purified by gel filtration on a Sephadex G25 (1 x 45 cm) column that was eluted with RIA buffer. Fifty fractions (1 ml) were collected and 5 μl of each fraction were counted for radioactivity.

Lactoperoxidase iodination method based on Von Schenk (172) was done by mixing, in order, glucagon, NaI^{125}, 20 μl of 0.015 M TRIS buffer (pH 10), 15 μg lactoperoxidase in 5 μl 0.4 M phosphate buffer and 5 μl (0.003%) hydrogen peroxide. Reaction was allowed for 45 seconds before the addition of 0.2 ml transfer-inhibition solution (0.01 M potassium phosphate dibasic, 0.1 M sodium nitrite, 0.1% BSA, 0.1 M sodium chloride, 0.1 M sodium iodide). Reaction mixture was purified by gel filtration as described for iodinations done by the chloramine T method.

Analysis of Iodinated Glucagon

Three different methods were used to identify the chromatography fractions containing [^{125}I]glucagon that were the most suitable for use in RIA. Fractions from the

start to the end of the [^{125}I]glucagon peak were tested. First, the percentage of ^{125}I incorporated into glucagon was determined by protein precipitation using 3 ml of 20% trichloroacetic acid (TCA) and 100 μl of each fraction. The mixtures were centrifuged at 1,500 x g for 30 min at 4°C and the precipitate obtained was counted for radioactivity. Second, the immunoreactivity of [^{125}I]glucagon tracer was tested by coincubating aliquots of the fractions with excess amount (100 μl) of C-terminal glucagon antisera (1:400 id) diluted in assay buffer for 1 h at room temperature. Two hundred microliters of goat anti-rabbit gamma globulin (1:250 id) were added and tubes incubated for 1 h at room temperature and then for 16 - 24 h at 4°C. Incubation ended with the addition of 3.8 ml cold rinse buffer and centrifugation at 1,500 x g for 45 min at 4°C. Percentage of antibody-bound [^{125}I]glucagon was determined by counting radioactivity of the precipitates. Finally, this latter approach of assessing the immunoreactive quality of tracer in fractions obtained from chromatography of the iodination mixture was repeated as described except that the RIA-working dilution (1 : 12,000 id) of the C-terminus antisera was used instead of excess antisera.

RESULTS

Effect of RIA-separation Procedure on the Molecular Profile of IRG

The molecular profile of IRG in chromatographed sheep plasma differed according to the procedure used in the RIA to separate antibody-bound and free [^{125}I]glucagon (Figure 7). RIA using the double-antibody separation procedure measured three major IRG peaks at the void volume (0 Kd), 0.15, and 0.72 Kd with molecular weights of approximately $\geq$145, 65 and 3.1 kDa, respectively. When PEG was used to separate antibody-bound and free [^{125}I]glucagon in the RIA, only two IRG peaks were detected at 0.15 and 0.72 Kd, respectively.

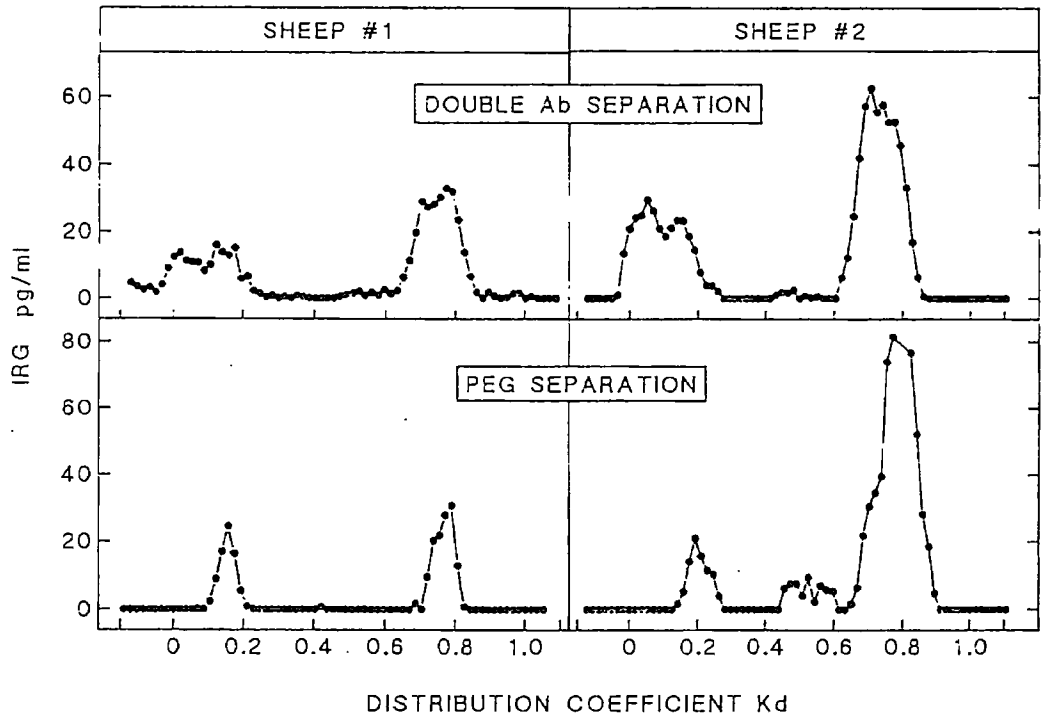

Figure 7. Molecular profile of IRG in chromatographed sheep plasma as measured by glucagon RIA using double-antibody or PEG to separate antibody-bound and free [^{125}I]glucagon. Ten milliters of sheep plasma were chromatographed on a Sephacryl 100 high resolution (S100HR ; 2.6 x 100 cm) column. Y-axis values are pg/ml of fraction eluent. Molecular weights of protein peaks eluting at void volume, 0.15 and 0.72 Kd were approximately $\geq$145, 61 and 3.1 kDa, respectively. Equation of the column calibration curve was log Y = -2.31X + 5.16; r = 0.994

Efficacy of PEG Extraction in Removing the Large Immunoreactive Plasma Proteins

The PEG-extraction procedure effectively removed all the large immunoreactive proteins from plasma (Figure 8). Charcoal absorption of plasma successfully absorbed all glucagon without affecting the quantity of the large immunoreactive proteins. The IRG concentration of plasma fell from 118 pg/ml to 56 pg/ml after charcoal absorption. Chromatography also showed that the IRG content of charcoal-absorbed plasma represented only the large immunoreactive proteins. PEG extraction of charcoal-absorbed plasma completely removed all measurable IRG.

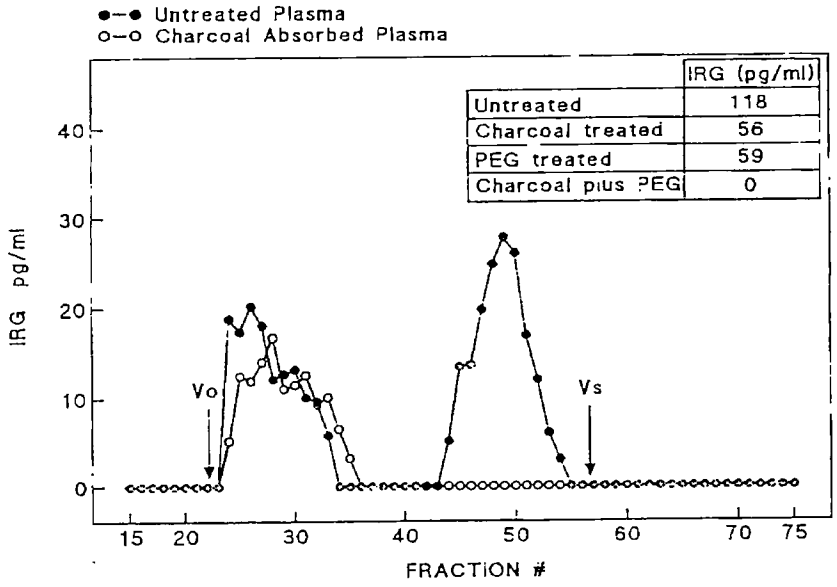

Figure 8. Removal of large immunoreactive proteins by PEG extraction of plasma. Sheep plasma (5 ml) was chromatographed on a Sephadex G50-50 (1.5 x 80 cm) column before (●-●) and after (o-o) charcoal absorption of the plasma. Y-axis values are pg/ml fraction eluent. Insert table : total IRG concentration of untreated plasma included that due to glucagon and the large molecular-weight immunoreactive proteins. IRG concentration of charcoal-absorbed plasma represented the concentration of only the large immunoreactive proteins in the sample. The difference in IRG concentration between untreated and charcoal-absorbed plasma hence represented the concentration of the large immunoreactive proteins. IRG concentration of PEG-extracted plasma represented the concentration of glucagon alone. PEG extraction of glucagon-free plasma (charcoal-absorbed) measured zero IRG concentration, therefore showing that PEG-extraction procedure effectively removed 100 % of the large immunoreactive proteins in plasma.

Validation of RIA for Measuring Glucagon in PEG-Extracted Plasma

Accuracy of the glucagon radioimmunoassay is shown in Figure 9. Linear regression analysis of the mean data indicated that the glucagon RIA essentially measured 100% of the glucagon in PEG-extracted plasma because the regression equation of the recovery curve was y = 0.978X - 11 (r = 0.992).

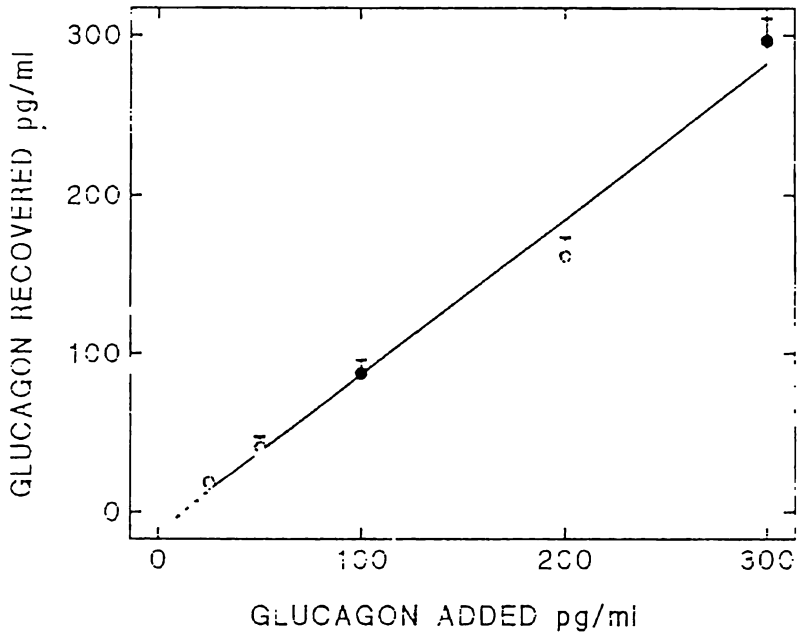

Figure 9. Accuracy of measuring glucagon in PEG-extracted plasma by RIA employing PEG to separate antibody-bound and free [^{125}I]glucagon. Average percentage recovery of the glucagon standard was 98 %. Regression equation of the recovery curve was y = 0.978X - 11 ; r = 0.992.

Recovery of [^{125}I]glucagon due to the PEG-extraction procedure itself was assessed by determining the radioactivity in the supernatant of PEG-extracted plasma (n = 3) that contained 50,000 CPM of [^{125}I]glucagon. Approximately 97 ± 0.9% of [^{125}I]glucagon was recovered in the PEG-extracted plasma.

Precision of the RIA in quantifying glucagon in PEG-extracted plasma is summarized in Table 2. The intra-assay CV ranged from 4 to 9% for plasma pools containing low, medium, and high concentration of glucagon. Interassay CV for another three plasma pools containing low, medium and high concentration of glucagon ranged from 8 to 15%.

Table 2. *Intra- and inter-assay precision for RIA measurement of glucagon in PEG-extracted plasma*

Precision	Plasma Pool	Glucagon (pg/ml)	CV (%)
Intra-assay	A	68.8 ± 1.9	6.0
	B	90.8 ± 2.2	5.5
	C	136.2 ± 2.8	9.1
Inter-assay	D	45.8 ± 1.9	9.2
	E	87.8 ± 3.2	8.2
	F	199.6 ± 13.9	15.6

PEG was used to separate antibody-bound and free [^{125}I]glucagon in the RIA. Glucagon values shown were as measured in PEG-extracted plasma pools and represented the mean (± SE) of 5 repeated measurements.

The combined imprecision in the system due to PEG-extraction and intra-assay ranged from 5-18% and averaged 11.1% (Table 3). The difference between the average intra-assay CV (6.7%) in Table 2 and the combined CV due to PEG extraction and intra-assay variability (11.1%) in Table 3 should represent the variability due to PEG extraction alone.

Table 3. *Combined imprecision in the RIA due to PEG-extraction and assay variability*

Plasma Pool	Glucagon (pg/ml)	CV (%)
Low	20.9 ± 1.17	17.7
Medium	49.4 ± 1.67	10.7
High	158.5 ± 2.01	5.0

Values are means (± SE) of ten observations. Each plasma pool was extracted ten times and the resultant supernatants were assayed in duplicate in the RIA.

Serial dilutions of PEG-extracted plasma and standard curves run in the presence and absence of PEG were parallel to each other (Figure 10). The presence of PEG did not affect the standard curve. Serial dilution of plasma resulted in binding inhibition curves that were parallel to both standard curves, which indicated that the same substance in standards and extracted plasma was being measured.

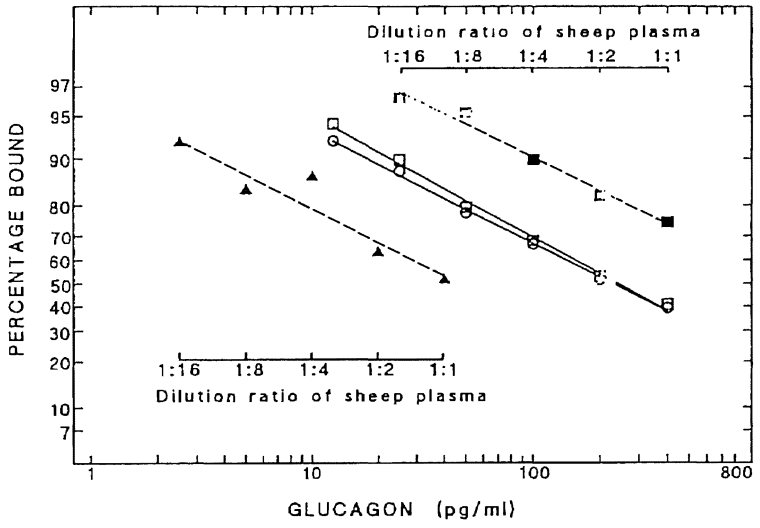

Figure 10. Binding inhibition curves for glucagon standards and PEG-extracted plasma that contained medium (~ 85 pg/ml; □ □) and high (~ 200 pg/ml; ▲-▲) concentrations of glucagon. Glucagon standard curves were assayed in the absence (□-□) or presence (○-○) of equivalent amounts of PEG (12.5%) as found in extracted plasma. PEG-extracted plasma containing 12.5% PEG was assayed before (1:1) and after its serial dilution (1:2, 1:4, 1:8, 1:16) in assay buffer.

The glucagon antisera used in this experiment did not crossreact significantly with potentially interfering compounds normally found in or added to sheep blood (Table 4, J.P. McCann, unpublished data). Interestingly, the crossreactivity with IgG was very low but IgG could interfere the radioimmunoassay because the plasma concentration of IgG is about 10^9-times greater than the plasma glucagon concentration.

Table 4. *Cross-reactivities of compounds in the glucagon radioimmunoassay*

Compound	Relative Activity*
Glucagon (bovine; 1.05 U/mg)	1.0000
Glucagon-Like Peptide 1	< 0.00001
Secretin	< 0.00001
Vasoactive Intestinal Polypeptide	< 0.00001
Gastric Inhibitory Polypeptide	< 0.00001
Gastrin I and II	< 0.00001
Cholecystokinin	< 0.00001
Insulin (ovine)	< 0.00001
Insulin (bovine)	< 0.0001
Proinsulin	< 0.00001
Somatostatin	< 0.00001
Pancreatic Polypeptide	< 0.00001
Calcitonin	< 0.00001
Parathyroid Hormone	< 0.00001
Calcium-Binding Proteins	< 0.00001
Growth Hormone	< 0.00001
Prolactin	< 0.00001
Thyroid Stimulating Hormone	0.00003
Luteinizing Hormone	0.00005
Globulins (sheep; cow; dog)	< 0.00000001
Immunogamma Globulins (sheep; cow; dog)	< 0.00000001
Albumin (ovine; bovine)	< 0.00000001
Benzamidine	< 0.0000001
Heparin	< 0.00000001

*Ratio of the concentration of glucagon to the concentration of the potentially interfering compound needed to inhibit binding of tracer to antibody by 50 percent (McCann, J.P., unpublished data).

Sensitivity of the assay system was 5.35 pg/tube or 27 pg/ml of plasma. Therefore, this RIA procedure can accurately quantify glucagon in as little as 200 μl of PEG-extracted sheep plasma.

Immunological Properties of the Large Immunoreactive Proteins

A single peak of [^{125}I]glucagon was detected in the chromatograph of [^{125}I]glucagon in RIA buffer. Mixing [^{125}I]glucagon with diluted sheep plasma did not change the magnitude or position of the [^{125}I]glucagon peak (Figure 11). Because data showed the absence of glucagon-binding protein, chromatography of unlabelled glucagon-[^{125}I]glucagon-plasma mixture was not done.

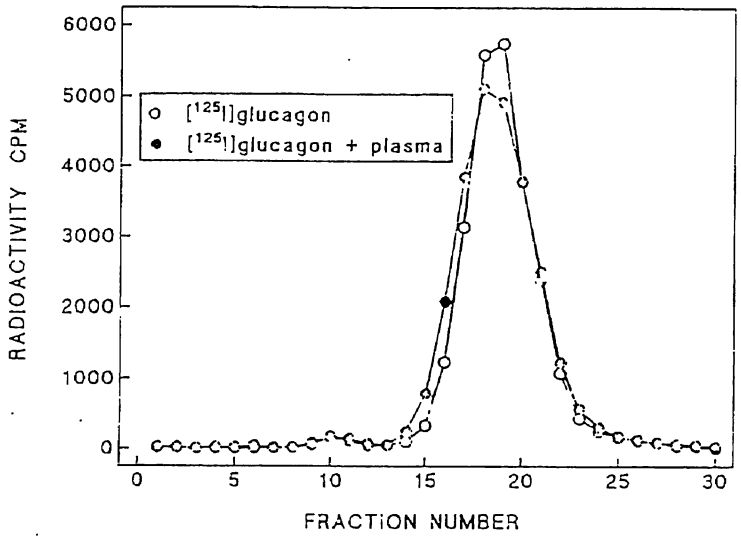

Figure 11. Test for the presence of a plasma glucagon-binding protein. Radioactivity counts were obtained from the chromatography of [^{125}I]glucagon previously incubated with buffer alone or buffer plus sheep plasma.

Incubation of [^{125}I]glucagon with the 61 kDa protein resulted in a slight increase in radioactivity in fractions eluting between the void volume and [^{125}I]glucagon. Incubating [^{125}I]glucagon with the aliquots of fractions containing the $\geq$145 kDa and 61 kDa proteins did not produced any change in the magnitude or position of the [^{125}I]glucagon peak (Figure 12).

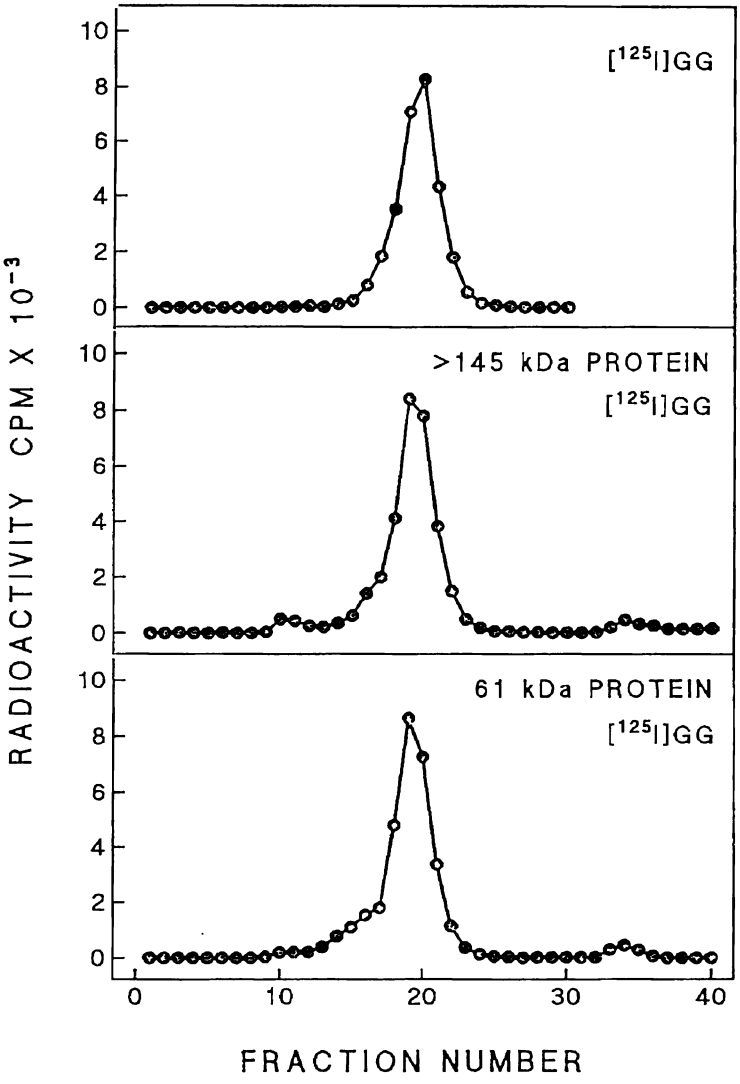

Figure 12. Chromatographs of [^{125}I]glucagon alone (top panel), [^{125}I]glucagon preincubated with the ≥ 145 kDa protein (middle panel), and [^{125}I]glucagon preincubated with the 61 kDa protein (bottom panel). All chromatographs were obtained from Sephacryl 100 HR (1 x 25 cm) column, void volume and bed volume of the column were marked by blue dextran (BD) and vitamin B_{12} (vit B), respectively. Fraction (1 ml) collected were counted for radioactivities.

Chromatography of the [^{125}I]glucagon-antisera showed two distinct radioactive peaks. The earlier eluting peak represented the antisera-bound [^{125}I]glucagon and the second one represented free [^{125}I]glucagon. Addition of the ≥145 kDa or 61 kDa proteins to the antisera-[^{125}I]glucagon mixture did not change the chromatographic profile of the [^{125}I]glucagon-antisera mixture (Figure 13).

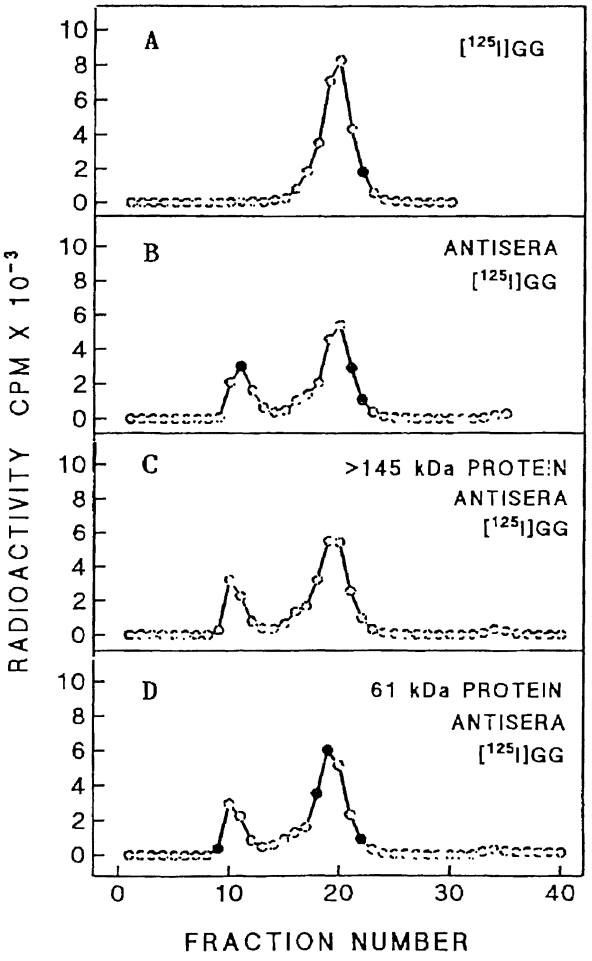

Figure 13. Chromatographs of [^{125}I]glucagon alone (panel A), [^{125}I]glucagon preincubated with antisera (panel B), [^{125}I]glucagon preincubated with antisera plus the ≥145 kDa protein (panel C), and [^{125}I]glucagon preincubated antisera plus the 61 kDa protein (panel D). All chromatographs were obtained from Sephacryl 100 HR (1 x 25 cm) column, void volume and bed volume of the column were marked by blue dextran (BD) and vitamin B_{12} (vit B), respectively. Fraction (1 ml) collected were counted for radioactivities.

The attempt to chromatograph sheep IgG and demonstrate that sheep IgG interfered in glucagon RIA was not entirely successful. Although chromatography of 202 mg of sheep IgG in 15 ml RIA buffer showed an IRG peak at the void volume (Figure 14), the result from this single chromatographic run was questionable because the column flow rate was inconsistent and faster than normal. Nevertheless, the glucagon peak eluted at its proper position and the peak of immunoreactivity at the void volume could only be due to the IgG.

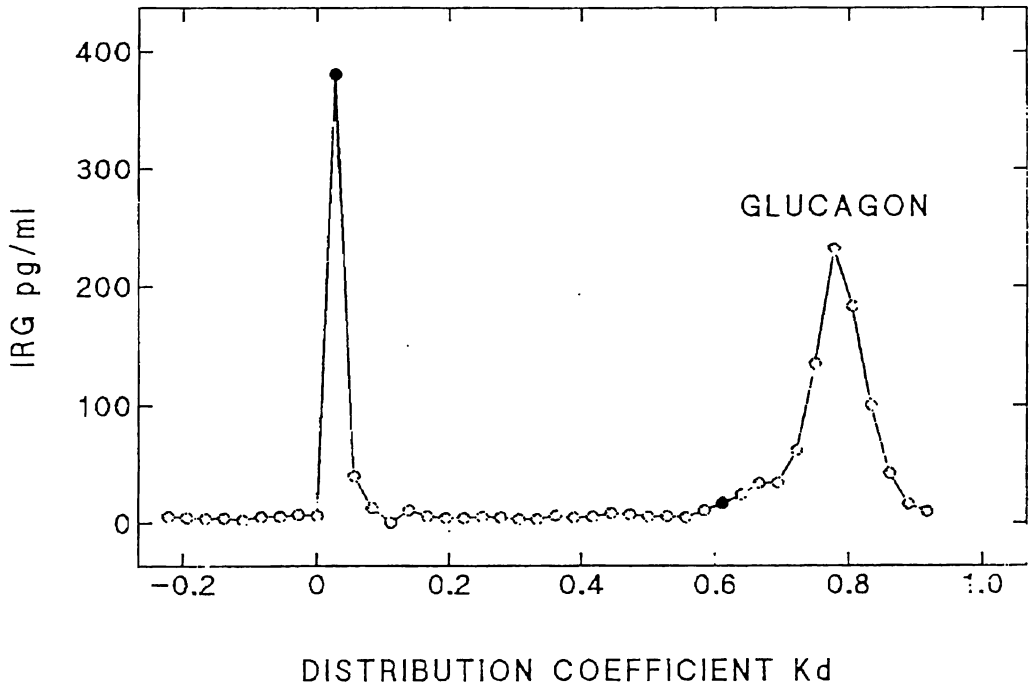

Figure 14. Chromatograph of 202 mg sheep IgG and 32 ng glucagon in RIA buffer. Sample (15 ml) was chromatographed on a Sephacryl 100 HR (2.6 x 10 cm) column, and fractions collected were measured for IRG by RIA. Void voume and bed volume were marked by blue dextran and vitamin B_{12}, respectively.

Glucagon Iodination

Chromatographic profiles of [^{125}I]glucagon prepared by the chloramine T (pH 7.4 and pH 10) and lactoperoxidase methods are summarized in Figure 15. All three iodination methods generated similar chromatographs that contained a single prominent protein peak of [^{125}I]glucagon and an I^{125} peak that eluted at the bed volume. A small peak of radioactive material consistently was observed immediately before the [^{125}I]glucagon peak in the lactoperoxidase method. Percentage precipitable counts obtained using TCA and excess amounts of antibody were directly proportional to the radioactivity content of the fraction. Therefore, according to the TCA and excess antibody test, the fractions that had the highest radioactivity should be the most suitable for use as tracer in the glucagon RIA. Based on precipitated counts obtained using the RIA-working dilution of antisera, however, the fraction containing the most radioactivity (i.e., peak radioactive fraction) was not the most suitable for use in the RIA; this latter approach identified the radioactive fraction eluting at the tail of the [^{125}I]glucagon peak as the most suitable for use as tracer in the RIA.

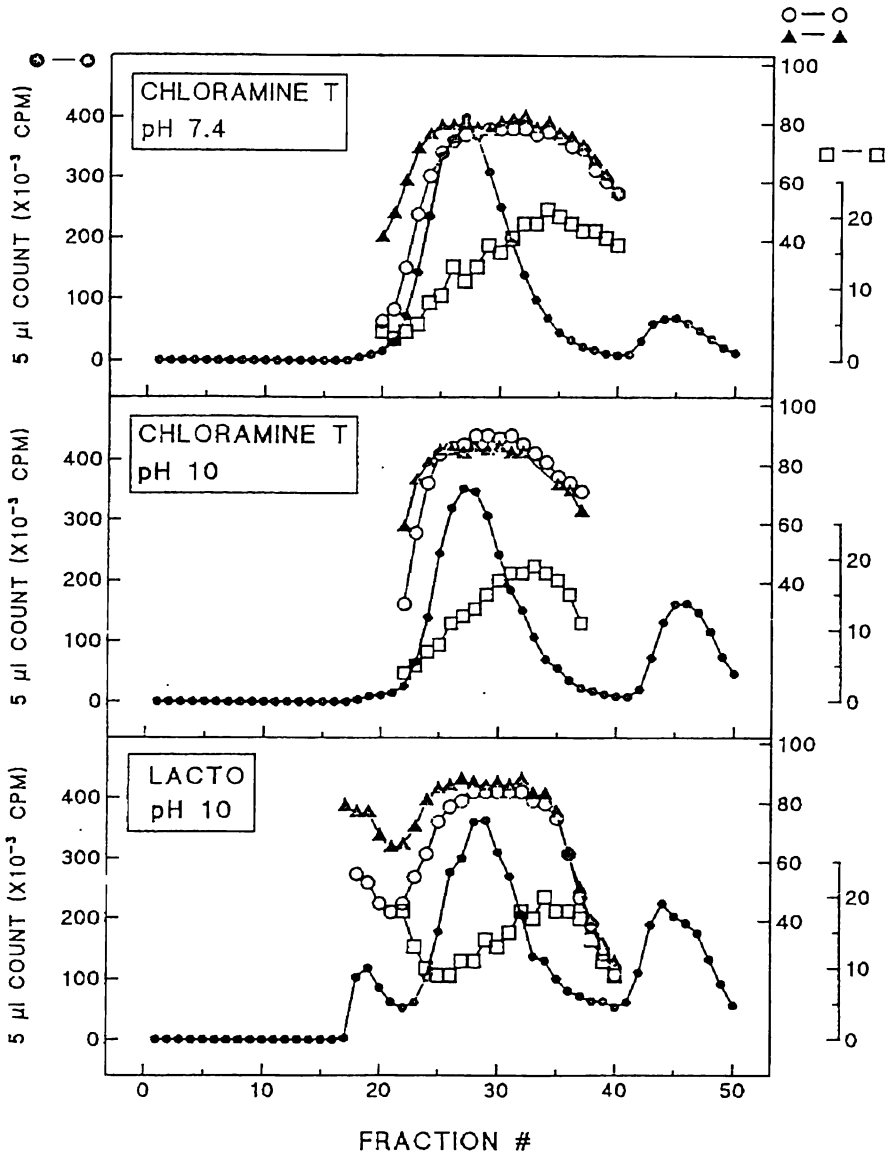

Figure 15. Comparison of glucagon iodination procedures and tests of suitability of [^{125}I]glucagon for use in RIA. Bovine glucagon was iodinated using chloramine T (pH 7.4 and pH 10) and lactoperoxidase (pH 10). Each panel shows radioactive counts obtained after chromatography of the iodination mixture (●-●) and the precipitable counts obtained using excess TCA (▲-▲), excess antibody (○-○) and antibody at RIA-working dilution (□-□). Data shown are results of single iodinations that represent typical results for 20 chloramine T iodinations at pH 7.4, 3 chloramine T iodinations at pH 10, and 9 lactoperoxidase iodinations at pH 10. The peak counts of [^{125}I]glucagon eluted at approximately fraction 27 and ^{125}I eluted at approximately fraction 45.

The fractions with the greatest amount of radioactivity that was precipitated by TCA or the two dilutions of antisera were tested further by performing binding inhibition curves for each of these three fractions (Figure 16).

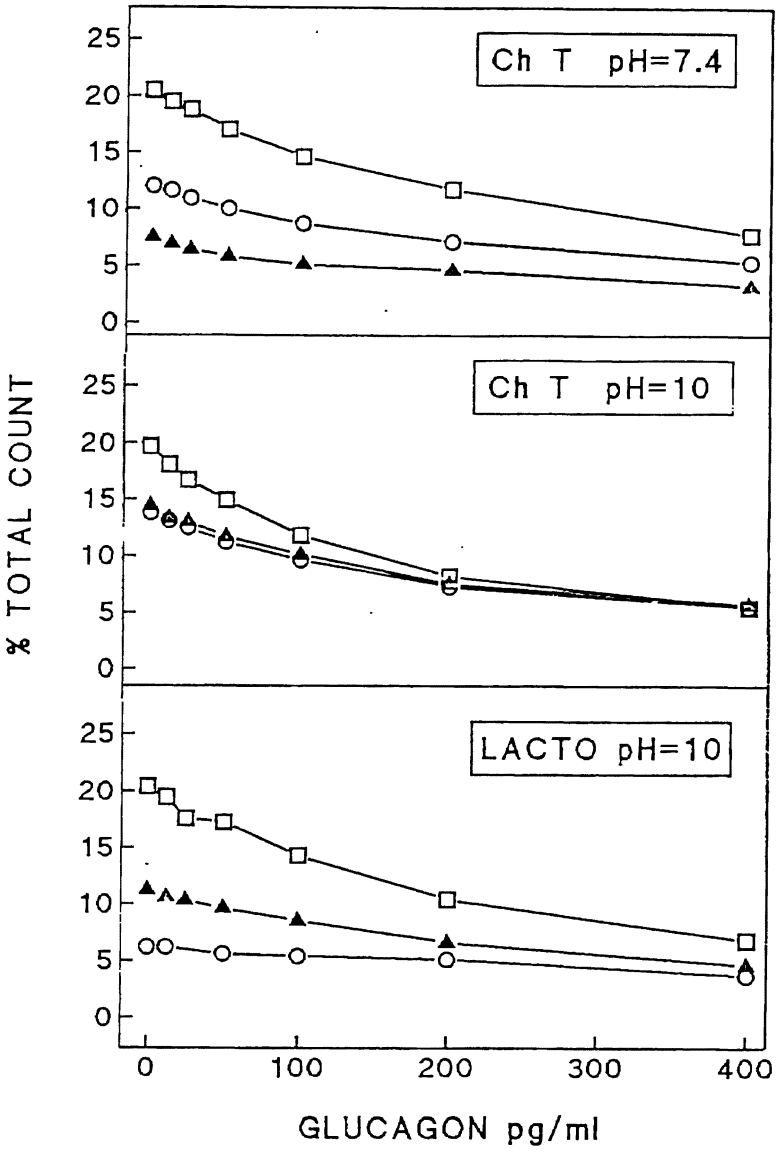

Figure 16. Binding inhibition curves for [^{125}I]glucagon prepared using chloramine T (pH 7.4 or 10) or lactoperoxidase (pH 10) iodination methods. Binding of [^{125}I]glucagon to antisera was inhibited by increasing concentrations of glucagon standard. Fractions from the chromatographic profile of each iodination mixture were selected for use as tracer glucagon in this experiment. TCA (▲-▲), and excess (○-○) and working dilutions (□-□) of the glucagon antisera were used to precipitate [^{125}I]glucagon in the fractions collected and the fraction containing the highest precipitable count provided the [^{125}I]glucagon tracer that was used to generate the binding-inhibition curves. The binding characteristics of each curve are summarized in Table 5.

The 'best' tracer fractions as determined by percentage precipitability by TCA, excess antibody, and antibody at working dilution produced standard curves with different slopes (sensitivity) and % total counts bound (Table 5). Regardless of the iodination environment, tracers selected according to percent precipitability by antibody at the RIA-working dilution always provided the best standard curve for glucagon radioimmunoassay.

Table 5. *Performance of glucagon standard curves using selected [^{125}I]glucagon tracers*

Parameters	Iodination Methods	Precipitation Procedure		
		TCA	Excess Ab	Working Ab
NSB (% TC)	Ch T (pH 7.4)	3.0	2.7	1.6
	Ch T (pH 10)	1.8	1.8	1.8
	Lacto (pH 10)	2.2	1.4	1.7
B_0 (% TC)	Ch T (pH 7.4)	7.2	11.7	20.1
	Ch T (pH 10)	14.2	14.2	20.9
	Lacto (pH 10)	9.6	6.4	19.7
Slope	Ch T (pH 7.4)	-1.86	-2.29	-2.06
	Ch T (pH 10)	-2.06	-2.06	-2.13
	Lacto (pH 10)	-1.90	-1.35	-1.36
ED (80) pg/ml	Ch T (pH 7.4)	44	66	62
	Ch T (pH 10)	53	53	47
	Lacto (pH 10)	84	49	55
ED (50) pg/ml	Ch T (pH 7.4)	274	300	274
	Ch T (pH 10)	246	246	215
	Lacto (pH 10)	452	557	230
ED (20) pg/ml	Ch T (pH 7.4)	1729	1360	1215
	Ch T (pH 10)	1134	1134	998
	Lacto (pH 10)	2030	6427	1194

Values for each method are from single iodinations that represent the typical results for 20 chloramine-T iodinations at pH 7.4, 3 chloramine-T iodinations at pH 10, and 9 lactoperoxidase iodinations at pH 10. TC, total counts added to assay tube; NSB, non-specific-bound counts; B_0, maximum antibody binding of [^{125}I]glucagon in the absence of competition due to glucagon standard; ED(80), ED(50), and ED(20) are concentrations of glucagon required to displace 80%, 50%, and 20% of the B_0 counts, respectively.

DISCUSSION

Quantification of plasma glucagon by RIA is complicated by the presence of large molecular weight plasma proteins that express glucagon-like immunoreactivity (166, 177). There are various methods to remove these large immunoreactive proteins from plasma prior to RIA measurement of glucagon concentrations. Attempts to physically separate the large immunoreactive proteins using ultrafiltration membranes (Amicron, Beverly, MA) were unsuccessful because more than 90% of unlabelled glucagon and [^{125}I]glucagon were adsorbed to the membrane (data not shown). Separation of the large immunoreactive proteins using organic solvents such as ethanol (67, 79) and acetone (173) essentially removed all the large immunoreactive proteins and allowed 75 to 80% recovery of glucagon in the extracted plasma. This study demonstrated that PEG extraction of sheep plasma effectively removed 100% of the large immunoreactive plasma proteins and allowed 97% recovery of glucagon. In comparison to the ethanol and acetone extraction methods, the PEG-extraction procedure is simpler and it provides a more precise and accurate quantification of plasma glucagon. Smaller quantities of PEG (150 μl of 35% PEG) can be mixed with plasma (850 μl) to minimize dilution of the plasma glucagon (data not shown).

This study showed that complete removal of the large immunoreactive proteins was essential before essential glucagon could be measured accurately by RIA. The biochemical properties of the large immunoreactive proteins are not known. Using the technique of gel chromatography, Weir (176) showed that the large immunoreactive proteins were composed of at least two proteins that eluted from the column with molecular weights corresponding to those of the gamma globulin (146 kDa for IgG_1, IgG_2

and IgG_4; 170 kDa for IgG_3) and bovine serum albumin (66 kDa) markers. Von Schenck (170) showed that human IgG is measured as IRG by the glucagon RIA. Human plasma that was immunoadsorbed with protein A to remove IgG (the IgG_1, IgG_2 and IgG_4 subclasses did not contain measurable amount of the large immunoreactive proteins by glucagon RIA (170). Von Schenck (170) also demonstrated that IgG and the constant portions (Fc) of IgG, but not the antigen-binding portion (Fab), were measured as IRG in the glucagon RIA.

In agreement with results in humans (176), two distinct protein peaks of molecular weight $\geq$145 and 61 kDa were detected when sheep plasma was chromatographed and the fractions measured by double-antibody RIA. However, the larger ($\geq$145 kDa) protein peak was not detectable in these same fractions when PEG was used instead of goat anti-rabbit IgG to separate antibody-bound and free [^{125}I]glucagon in the RIA. This suggested that the $\geq$145 kDa peak may bind [^{125}I]glucagon and that the bound-complex was precipitated by PEG. As a consequence, the higher than expected radioactive counts in the precipitated pellet would result in an artifactually lower, or zero, concentration reading from the standard curve. When double antibody-RIA was used, the $\geq$145 kDa protein-[^{125}I]glucagon complex would not be recognized by the goat anti-rabbit IgG. However, the $\geq$145 kDa protein would prevent some [^{125}I]glucagon from interacting with the glucagon antisera, and this potentially could lower the radioactivity count in the precipitated pellet. The lower radioactive count would be measured as IRG.

The fact that a small void volume peak appeared in the chromatograph of [^{125}I]glucagon preincubated with the $\geq$145 kDa protein (Figure 12) suggested that the $\geq$145 kDa protein bound to glucagon to some extent. However the postulate that $\geq$145 kDa protein may binds glucagon was not supported by other preliminary results

demonstrating the absence of a sheep plasma-binding protein (Figure 11) and the inability of the ≥145 kDa protein to displace the antisera-[^{125}I]glucagon peak (Figure 13). Further studies of the properties and chemical identity of the large immunoreactive proteins is warranted.

On the other hand, the smaller immunoreactive protein (61 kDa) may behave as unlabelled glucagon that competes with glucagon and [^{125}I]glucagon for binding to the glucagon antisera. The tracer displaced by the 61 kDa protein would not be precipitated by either goat anti-rabbit IgG or PEG. Therefore the 61 kDa protein would interfere to the same degree in RIAs using the double-antibody or PEG-separation methods.

If indeed the ≥145 protein binds [^{125}I]glucagon and the 61 kDa protein competes with glucagon for antisera binding, then RIA using reagents (e.g. acetone or ethanol) that precipitate the antibody-bound [^{125}I]glucagon should behave the same as RIA using the PEG-separation method. RIAs of unextracted plasma that use acetone, ethanol or PEG methods to separate antibody-bound and free [^{125}I]glucagon will measure a lower IRG concentration than that using a double-antibody separation procedure because the former do not measure the ≥145 kDa protein. However, RIA using a dextran-coated charcoal separation method was able to detect both a larger (160 kDa) and a smaller (68 kDa) immunoreactive protein equally as well as the double-antibody method (177). If the larger immunoreactive protein (≥145 kDa) actually binds [^{125}I]glucagon tracer, then the binding affinity may be weak enough that the charcoal is capable of stripping [^{125}I]glucagon from the ≥145 kDa protein. Although the actual characteristics of the ≥145 kDa protein are not well understood, the results of this experiment clearly show that the concentration of IRG in unextracted plasma would differ according to the procedure used in RIA to separate antibody-bound and free [^{125}I]glucagon. Therefore, removal of the large

immunoreactive proteins prior to the measurement of plasma glucagon by RIA is recommended.

The quality of iodinated hormones for use in RIA traditionally has been assessed by the percent of radioactive counts precipitable by TCA or excess antisera. This study showed that [^{125}I]glucagon selected according to those methods was not the best quality tracer for use in RIA. Typically, fractions containing [^{125}I]glucagon that were selected in this study by the TCA or excess antibody methods produced standard curves with low sensitivity. The RIA-working dilution of antisera identified the fractions containing the best quality tracer for RIA; these fractions, routinely always eluted 4 to 5 fractions after the radioactive-glucagon peak. The later eluting fractions may contain mainly monoiodoglucagon while the fractions around the radioactive glucagon peak may contain mainly diiodoglucagon. As suggested by Von Schenck (172), diiodoglucagon molecules are less immunoreactive and are more subject to iodination damage. This may be the reason why fractions containing the peak counts of [^{125}I]glucagon had apparently good immunoreactivity when tested with excess antisera but reduced immunoreactivity when tested using limited amounts of antisera.

In summary, this study validated a PEG-extraction method for removing the large immunoreactive proteins from plasma. Radioimmunoassays of unextracted plasma that use PEG to separate antibody-bound and free [^{125}I]glucagon will measure glucagon and the smaller (61 kDa) of the two large immunoreactive proteins, whereas the double-antibody procedure will measure glucagon and both immunoreactive proteins. Therefore double antibody and PEG separation procedures in RIA provide different total IRG values in unextracted plasma. Nevertheless, RIA of neat plasma using C-terminus specific antisera will not provide accurate concentrations of glucagon regardless of the RIA-

separation procedure. Complete removal of the large immunoreactive proteins is a necessary step for accurate measurement of plasma glucagon by RIA. Quality [^{125}I]glucagon was prepared by the chloramine T (pH 7.4 or pH 10) and lactoperoxidase (pH 10) methods, but the best tracer for RIA had to be identified by percentage precipitable counts obtained using limited amounts of antisera rather than excess antisera or TCA. Fractions identified in this manner consistently eluted at the tail of the radioactive glucagon peak.

Chapter IV

WHOLE BODY KINETICS OF GLUCAGON IN LEAN AND OBESE SHEEP

INTRODUCTION

The obese sheep model has experimental application to obesity and non-insulin-dependent diabetes mellitus (NIDDM) in humans because obese sheep display similar endocrine, metabolic and cardiovascular abnormalities as found in humans with obesity or NIDDM (6, 98, 100). Hyperinsulinemia, hyperglycemia and insulin resistance are consequences of dietary-induced obesity in sheep (100). Hyperinsulinemia in obese humans and obese sheep may coexist with hyperglucagonemia (33, 100, 150). Direct and indirect measurements have shown that greater-than-normal secretion rates of insulin were responsible for the hyperinsulinemia in obese cattle and sheep (103, 104). However, the kinetic basis for hyperglucagonemia in obese animals and humans is not known.

Regardless of body condition state, relative or absolute hyperglucagonemia was found in humans with insulin-dependent diabetes mellitus (IDDM) and NIDDM (129, 164). In nondiabetic people, fasting plasma concentrations of immunoreactive glucagon (IRG) were either normal (129), significantly elevated (102, 150), or nonsignificantly elevated (81) in obese compared with nonobese individuals. One extensive study showed that plasma IRG concentrations were similar in nondiabetic control subjects and in subjects with moderate or severe degrees of dietary obesity when frequent measurements were

made throughout the day (129). Such results suggest that glucagon dysfunction is lacking or is an infrequent finding in obese humans. This may be an erroneous conclusion because immunoreactive glucagon levels in peripheral plasma may not reflect the true concentration of glucagon in animals and humans.

Glucagon radioimmunoassays that are considered specific for glucagon actually measure IRG which is comprised of glucagon and large molecular weight plasma proteins with glucagon-like immunoreactivity (see Chapter 3). The extent of cross-reactivity of large molecular weight plasma proteins in the glucagon RIA depends on the source of glucagon antisera used (149). The relative abundance of these large reactive proteins in plasma may mask small differences in plasma levels of pancreatic glucagon. One study that measured plasma glucagon exclusive of the large immunoreactive proteins found that the peripheral plasma level of glucagon was slightly but significantly elevated in obese humans (150).

The objectives of these experiments were to determine how obesity affected glucagon kinetics in sheep. In addition, the plasma insulin, glucose and free fatty acid (FFA) responses to a high dose of glucagon stimulation were compared in lean and obese sheep because insulin and glucagon have reciprocal effects on the plasma glucose concentration and each can affect the secretion rate of the other hormone.

MATERIALS AND METHODS

Dietary Obese Sheep

Details of the feed intakes and body weight changes during the induction (dynamic) and maintenance (static) phases of dietary obesity in sheep were reported elsewhere (102). Briefly, obesity was induced in adult ewes by overfeeding with a pelleted hay-grain diet and this same diet was fed at a maintenance level to the lean (control) ewes. Static phase obesity was achieved after approximately 40-50 wk of ad libitum feeding.

Lean and obese Dorset ewes aged 3 to 5 yr were used in this experiment. Body weight in obese sheep (91 $\pm$ 1 kg) exceeded ($P<0.05$) that of lean (41 $\pm$ 1 kg) sheep. These differences in body weight were associated with significantly greater depths of subcutaneous fat, weights of internal fat depots, and an estimated percent body fat in the live animal of 20 to 26 percent in lean sheep and 34 to 40 percent in obese sheep (102). Lean and obese sheep were housed individually in pens in a room with constant light and temperature (21 $\pm$ 1°C). Water was available continuously and experiments were conducted when lean and obese sheep were at zero energy balance and equilibrium body weight.

Glucagon Injection and Sample Collection

Bovine glucagon (Lilly Research Laboratories, Indianapolis, IN) was dissolved in 0.01 M TRIS buffer (pH 9.8) and 0.5 ml of each animal's own sterile plasma immediately before injection. Glucagon (1 μg/kg) was injected (0830 h) via jugular catheter (Angiocath®, Becton Dickinson, Sandy, UT) into 20-h fasted sheep. Catheters were inserted percutaneously at least 16 h before each experiment. Catheters were flushed with 5 ml saline after glucagon injection. Blood samples (4 ml) were collected

into heparinized syringes at 2, 4, 6, 8, 10, 12, 15, 18, 20, 22, 25, 27, 30, 32, 35, 37, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110 and 120 min after glucagon injection in lean (n=8) and obese (n=8) ewes; additional blood samples (4 ml) were collected 20, 10 and 1 min before the glucagon injection. Blood was immediately dispensed into chilled polystyrene tubes containing benzamidine (10 mg) and heparin (200 U) that were held in an ice-water bath. Plasma was recovered by centrifugation (4°C) and stored at -20°C.

Plasma samples collected for the glucagon kinetic study were used to quantify plasma insulin, glucose and FFA responses to glucagon stimulation (n=8 per group). Measurements were made in samples collected 20, 10, and 1 min before and 4, 8, 12, 20, 25, 30, 40, 80, and 120 min after glucagon injection.

Analytical methods

Plasma insulin was measured by solid phase radioimmunoassay validated for use in sheep (105). Sensitivity of the assay was 6.0 pmol/L; intra-assay and interassay coefficients of variance (CV) were 8% and 14%, respectively. Plasma glucose concentration was determined by Trinder reagent as described previously (102); intra-assay and interassay CV were 0.5% and 4.5%, respectively. Plasma FFA concentrations were determined using an enzymatic colorimetric method described by McCann et al (102); intra-assay and interassay CV were 3% and 7%, respectively.

A double-antibody radioimmunoassay using polyclonal rabbit anti-glucagon serum was validated in this laboratory for measurement of glucagon in sheep (101). Intra-assay and interassay CV were 3% and 12%, respectively. Radioimmunoassays using this or Unger's 30K antisera provide equivalently specific measurement of plasma concentrations of pancreatic glucagon, although each system also measures large molecular weight (≥

61 kDa) immunoreactive components in plasma (102). Concentrations of glucagon in plasma after glucagon injection were determined by the double antibody procedure.

Plasma concentrations of glucagon plus the large immunoreactive proteins (i.e., IRG) were measured in the basal samples collected before glucagon injection. Glucagon concentrations alone were determined after plasma was treated with polyethylene glycol 8000 (PEG; Sigma, St Louis, MO). As described in Chapter 3, PEG treatment removes the large immunoreactive protein component of IRG and allows accurate quantification of glucagon in the supernatant of PEG-extracted plasma.

Separation of antibody-bound and free [^{125}I]glucagon (Diagnostic Products Corporation, Los Angeles, CA) in the RIA for quantification of IRG in unextracted plasma and pancreatic glucagon in PEG-extracted plasma was achieved using charcoal absorption (64). For each sample, extracted and unextracted plasma were run in the same assay. Recovery of bovine glucagon (Lilly, Indianapolis, IN) in PEG-extracted sheep plasma averaged 110 $\pm$ 5 percent when 100, 200 and 400 pg glucagon standard were added (50 μL) to plasma aliquots (1.95 ml) of three sheep before PEG extraction. In this paper, the term IRG will refer to the total immunoreactive glucagon as measured by RIA which includes immunoreactivity due to glucagon (3,500 Da) and large molecular weight ($\geq$61 kDa) plasma proteins; the term glucagon will be reserved for glucagon (3,500 Da) as measured by RIA in PEG-extracted plasma.

Calculations and statistical analysis

Concentrations of injected glucagon in each sheep were measured by subtracting the average concentration of IRG in three pre-injection samples from the IRG concentration

measured at each time point after glucagon injection. This is based on the observation that the concentration of the large immunoreactive proteins remained constant when plasma glucagon concentrations increased during an arginine-stimulated-rise in plasma IRG levels (see Chapter 5). For accurate calculation of glucagon secretion rate, it was assumed that the large immunoreactive component of IRG remained constant throughout the sampling period of this experiment.

Best fit of the relationship between log glucagon concentration in plasma and time was determined by computerized nonlinear regression analysis (P-Fit, Biosoft, Milltown, NJ) of all observations within each body condition group. Comparison of regression residual sum of squares and coefficients of determination showed that a bi-exponential equation best described the disappearance of injected glucagon and that glucagon kinetics were described best by an open two-compartment model (132, 147, 178). Bi-exponential equations then were fitted to individual sheep to provide eight estimates of glucagon kinetics in each body condition group. Glucagon kinetics were calculated from the biexponential equation $C(t) = A_0e^{-\alpha(t-t0)} + B_0e^{-\beta(t-t0)}$, where C(t) equals concentration of injected glucagon in plasma at time t. The individual kinetic variables for glucagon were defined (132, 178) as:

A_0 ; time-zero plasma concentration intercept of the distribution phase

B_0 ; time-zero plasma concentration intercept of the elimination phase

$k_1 = A_0B_0(\alpha-\beta)^2/(A_0+B_0)(A\beta+B\alpha)$; rate at which glucagon is transferred from the central compartment to the peripheral compartment

$k_2 = (A_0\beta+B_0\alpha)/(A_0+B_0)$; rate at which glucagon is transferred from the peripheral compartment back to central compartment

$k_3 = \alpha\beta/k_2$; rate at which glucagon is irreversibly lost from the central compartment

V_d = glucagon dose/(A_0+B_0) ; volume of distribution of injected glucagon

V_{dss} = $V_d[1+(k_1/k_2)]$; volume of distribution of injected glucagon at finite time when the amount of glucagon is equivalent in the central and peripheral compartments

$t_{1/2\alpha}$ = $0.693/\alpha$; half-life of the distribution phase

$t_{1/2\beta}$ = $0.693/\beta$; half-life of the elimination phase, i.e. biological half-life

MCR = $V_d k_3$; metabolic clearance rate

SR = MCR x basal plasma glucagon concentration ; estimated secretion rate of glucagon

Because the basal plasma glucagon concentration was measured at a peripheral site instead of the portal vein, the calculated secretion rate was actually the post-hepatic delivery rate of glucagon. Differences in glucagon kinetic variables and pancreatic secretory rates between lean and obese sheep were determined by Student's unpaired t test.

Differences between lean and obese sheep in glucagon-induced changes in plasma concentrations of insulin, glucose and FFA were determined by repeated measures analysis of Gill (53). Differences in basal concentrations of insulin, glucose and FFA between lean and obese sheep before glucagon injection were accounted for by determining the response area above basal for each of these variables. Area-under-the-curve (AUC) was calculated by trapezoidal method (P-Fit, Biosoft Company, Milltown, NJ). The AUC allows determination of the net response in insulin, glucose and FFA metabolism in lean and obese sheep after perturbation of the system by injection of a high stimulatory dose of glucagon. Student's unpaired t test determined differences in AUC between lean and obese sheep. Level of significance was 0.05 and values presented are means $\pm$ SE.

RESULTS

Whole body kinetics of injected glucagon

The plasma concentration-time curves for injected glucagon in lean and obese sheep were similar and described best by biexponential equations (Figure 17). Such results indicated that glucagon disappearance from plasma followed first order kinetics and that whole-body kinetics of injected glucagon were described adequately by an open two-compartment model (132, 178).

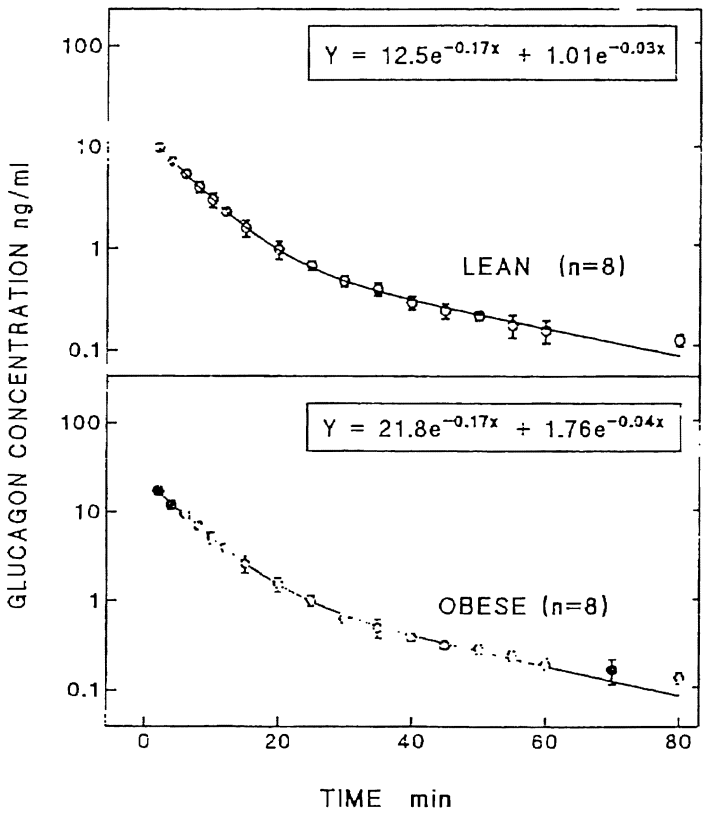

Figure 17. Plasma concentration-time curves of glucagon in lean and obese Dorset ewes injected iv (time 0) with 1 μg/kg bovine glucagon. Values are means ± SE and were obtained by subtracting basal (pre-injection) values of plasma IRG from plasma IRG levels after glucagon injection. Shown are the biexponential equations derived for the fit of the mean data points in each group. Note log scale of y-axis.

The half-life of glucagon during the distribution (~3 min) and elimination (~17 min) phases was unaffected by obesity (Table 6). However, time-zero plasma concentration intercept of the distribution phase (A_0) was about 80% greater ($P<0.05$) in obese than lean sheep.

Table 6. *Parameters of two-component-exponential equations describing disappearance of iv injected glucagon from plasma in lean and obese Dorset sheep*

Item	Phase	Lean	Obese
Intercept concentration (ng/ml)	Distribution(A_0)	14.1 ± 2.5	25.2 ± 3.5*
	Elimination (B_0)	2.8 ± 1.1	3.2 ± 1.0
Fractional removal rate (min^{-1})	Distribution(α)	0.25 ± 0.06	0.23 ± 0.03
	Elimination(β)	0.06 ± 0.02	0.08 ± 0.02
Half life (min)	Distribution ($t_{1/2\alpha}$)	3.2 ± 0.6	3.4 ± 0.5
	Elimination ($t_{1/2\beta}$)	18.0 ± 4.5	16.7 ± 4.8

Values are means ± SE (n = 8 per group).
*P < 0.05, different from lean value.

Glucagon kinetics (k_1, k_2, k_3) were similar in lean and obese sheep. Absolute Vd was similar in lean (3.38 ± 0.4 L) and obese (4.10 ± 0.49 L) sheep, but it was greater in lean than obese sheep when indexed to body weight (Table 7). The calculated absolute secretion rate of glucagon in obese sheep was nearly twice that of lean sheep (Table 7). Secretion rates of glucagon were similar ($P>0.05$) in lean and obese sheep when expressed relative to unit or metabolic body weight. The absolute MCR was approximately 34% greater ($P<0.1$) in obese than lean sheep. However, the MCR of glucagon was significantly greater in lean than obese sheep when expressed relative to unit or metabolic body weight.

Table 7. *Estimates of glucagon kinetics in lean and obese Dorset sheep derived from an open two-compartment model*

Item	Lean	Obese
k_1 (min^{-1})	0.532 ± 0.13	0.584 ± 0.09
k_2 (min^{-1})	0.071 ± 0.02	0.092 ± 0.02
k_3 (min^{-1})	0.187 ± 0.04	0.188 ± 0.03
V_d ($ml \cdot 100\ g^{-1}$)	8.3 ± 1.1	4.5 ± 0.6†
V_{dss} (L)	33.8 ± 9.0	37.0 ± 4.9
MCR ($ml \cdot min^{-1}$)	536 ± 35	721 ± 87
MCR ($ml \cdot kg^{-1} \cdot min^{-1}$)	13.2 ± 0.9	7.9 ± 1.0†
MCR ($ml \cdot kg^{-0.75} \cdot min^{-1}$)	33.5 ± 2.5	24.3 ± 2.9*
SR ($ng \cdot min^{-1}$)	30.1 ± 3.2	55.0 ± 8.4*
SR ($ng \cdot kg^{-1} \cdot min^{-1}$)	0.75 ± 0.1	0.61 ± 0.1
SR ($ng \cdot kg^{-0.75} \cdot min^{-1}$)	1.9 ± 0.2	1.7 ± 0.2

Values are means ± SE (n = 8 per group) calculated (see Methods) from components of biexponential equations describing disappearance of iv injected glucagon from plasma.
* $P < 0.05$, different from lean value.
† $P < 0.01$, different from lean value.

Metabolic responses to glucagon

Changes in plasma concentrations of insulin, glucose and FFA after iv injection of glucagon were determined to assess whether obesity affected pancreas, liver and adipose responses to acute glucagon stimulation. Basal plasma concentrations of IRG, insulin and glucose, but not FFA, were greater ($P<0.05$) in obese than lean sheep (Table 8).

Table 8. *Basal plasma concentrations of IRG, glucagon, insulin, glucose and FFA in lean and obese Dorset sheep*

Item	Lean	Obese
IRG (ng/ml)	76.1 ± 4.2	101 ± 6.7†
Glucagon (pg/ml)	56.0 ± 3.5	71.2 ± 4.9*
Insulin (μU/ml)	5.3 ± 1.3	18.6 ± 3.7†
Glucose (mg/dL)	49.8 ± 1.6	55.9 ± 1.1*
FFA (μM)	356 ± 47	397 ± 51

Values are mean ± SE for an average of 3 observations in each animal; n = 8 per group. Glucagon values measured in plasma extracted with PEG to remove large molecular weight plasma proteins that display glucagon-like immunoreactivity (see Methods).
*P < 0.05, different from lean value.
†P < 0.01, different from lean value.

Glucagon injection resulted in rapid and significant increases in the plasma concentrations of insulin, glucose and FFA in both groups of sheep (Figure 18). The increase in insulin concentrations was more pronounced in obese than in lean sheep, even after considering for the difference in basal insulin concentration between the lean and obese sheep. The plasma concentration-time curves for plasma glucose and FFA responses to injected glucagon were similar in lean and obese sheep.

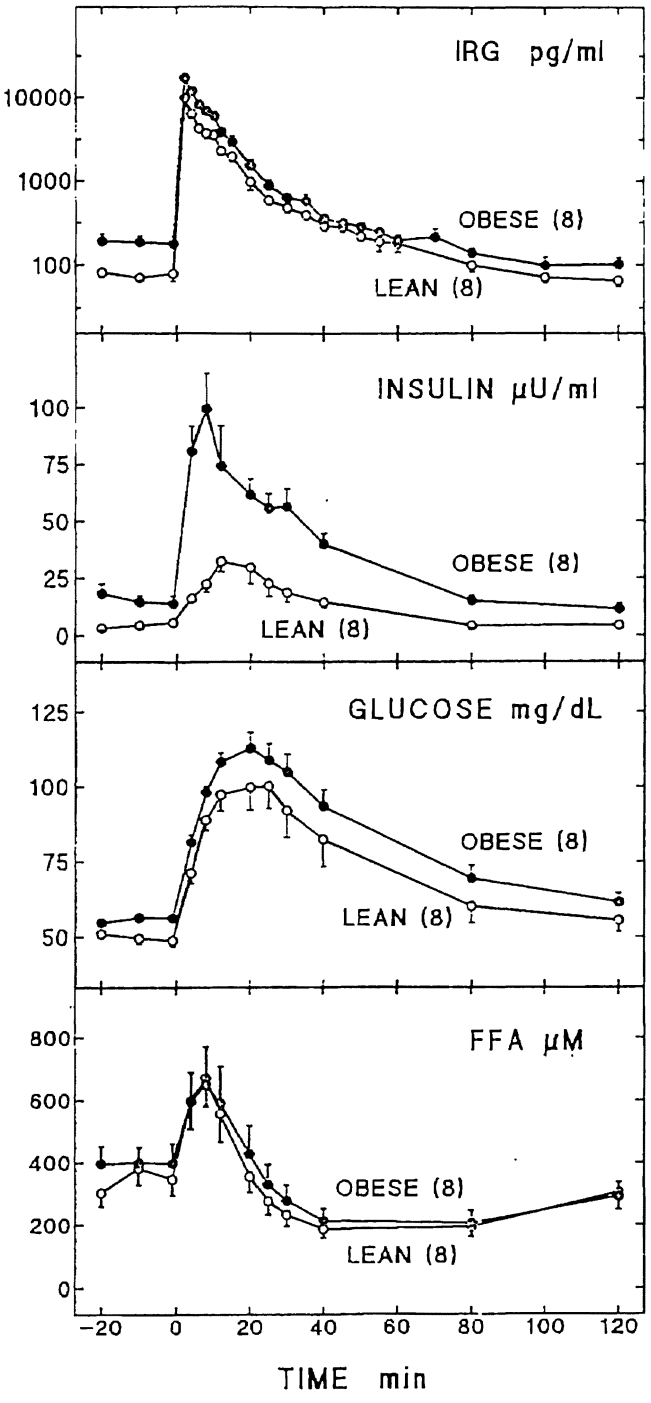

Figure 18. Mean (± SE) plasma concentrations of IRG, insulin, glucose and FFA in lean and obese sheep before and after the iv injection (time 0) of 1 μg/kg bovine glucagon.

AUC were calculated in order to determine more clearly the effect of obesity on the insulin, glucose and FFA responses to stimulation by an approximate fifteen-fold increase in the physiological concentration of plasma glucagon. This manoeuvre corrected for differences in the basal concentrations of plasma variables between lean and obese sheep. The acute (0 to +20 min) and total (0 to +120 min) insulin response areas were greater ($P < 0.05$) in obese than lean sheep, but those for glucose and FFA were not affected by body condition (Figure 19).

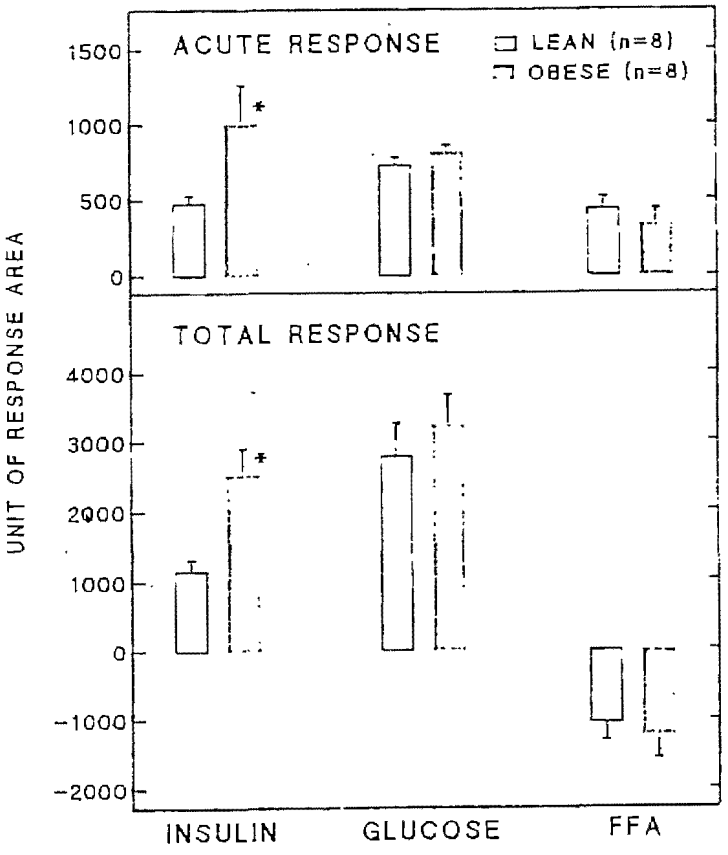

Figure 19. Acute (0 to +20 min) and total (0 to +120 min) response areas for glucagon-induced changes in plasma concentrations of insulin, glucose and FFA in lean and obese sheep. Positive response means that the net response of the system was an increase above basal (pre-injection) values. Values are means ± SE. Units of response area are $\mu U \cdot ml^{-1} \cdot min^{-1} \times 10^{-2}$ for insulin, $mg \cdot dL^{-1} \cdot min$ for glucose and $\mu M \cdot min^{-1} \times 10^{-2}$ for FFA.
* $P < 0.05$, different from lean value.

DISCUSSION

The kinetic basis for obesity-associated hyperglucagonemia in sheep was determined using compartmental kinetics of iv injected glucagon. A single pulse injection of glucagon should be a valid approach for multi-compartmental analysis of glucagon kinetics in animals (147). The whole-body kinetic behavior of injected glucagon in this study was described best by an open two-compartment model. Glucagon kinetics in humans (7), pigs (167), and domestic ruminants (37) have been described previously by an open two-compartment model. The model is composed of a central compartment, which presumably includes the vascular system, and a peripheral compartment. Each compartment represents multiple tissues and/or fluids that are lumped together within a compartment because of their similarity in kinetic handling of glucagon.

The kinetic behavior of iv injected glucagon essentially was similar in lean and obese sheep. As regards the plasma disappearance of injected glucagon, the only significant difference measured was that of a greater time-zero plasma concentration intercept for the distribution phase (A_0) in obese compared with lean sheep. Because blood and plasma volume per unit body weight decreases with increasing degree of obesity in sheep (94), a larger A_0 value was not an unexpected finding in obese compared with lean sheep given that glucagon dosage was proportional to body weight. For any time period, approximately 65 percent of the glucagon pool in the central compartment flowed to the peripheral compartment, with only 19 percent of the pool irreversibly leaving or being eliminated from the central compartment. The physiological identity of the peripheral compartment is not known; it may include the liver and kidney which are highly vascularized and very actively sequester glucagon in sheep, humans and rodents (11, 27, 78).

MCR indicates the hypothetical volume of plasma that loses its glucagon content each minute. The MCR of glucagon was significantly lower in obese than in lean sheep when expressed relative to unit or metabolic body weight. Comparing MCR of a given compound among different groups of animals as a measure of whole-body handling or removal of the compound will be worthwhile if the plasma concentrations of the compound are similar among groups of animals. In this study, the plasma concentrations of IRG in unextracted plasma and glucagon in extracted plasma were greater ($P < 0.05$) in obese than lean sheep. Therefore comparisons of actual rate of glucagon removed (ng/min) would be more reliable than the comparison of glucagon MCR per unit weight of lean and obese sheep.

Glucagon MCR of 8 to 13 $ml \cdot kg^{-1} \cdot min^{-1}$ in sheep in this study were similar to those of 9 to 11 $ml \cdot kg^{-1} \cdot min^{-1}$ in non-diabetic humans (7), 11 to 14 $ml \cdot kg^{-1} \cdot min$ in lean and obese humans (49), 12.6 $ml \cdot kg^{-1} \cdot min^{-1}$ in normal dogs (77), and 5 to 10.5 $ml \cdot kg^{-1} \cdot min^{-1}$ in normal sheep (26) and in lactating dairy cows with and without ketosis (37).

Relative fasting hyperglucagonemia seems to be a consistent finding in lean and obese patients with NIDDM (129, 164) but is an equivocal finding in obese nondiabetic subjects (73, 81, 127, 150, 180). One partial explanation for the inconsistency in detecting significant hyperglucagonemia in obese nondiabetic humans may be that hyperglucagonemia is only expressed in obese subjects with hepatic lipidosis (73). We have observed repeatedly a 20 to 100 percent increase in the fasting plasma IRG level in obese compared with lean sheep (100, 102, McCann unpublished data). In this study, fasting plasma IRG and glucagon levels were significantly greater in obese than lean Dorset ewes. Similar or increased levels of plasma IRG should indicate relative

hyperglucagonemia in obese animals that are hyperinsulinemic and mildly hyperglycemic because both insulin and glucose normally inhibit glucagon secretion by the pancreas (158, 164).

The kinetic mechanism responsible for the obesity-associated hyperglucagonemia in this study was that of a greater glucagon secretion rate in obese compared with lean sheep. Glucagon secretion rate was estimated indirectly and was approximately twice as great in obese than lean Dorset ewes that were fasted 20 h. These results suggest that increased secretion rates for glucagon may be associated with fasting hyperglucagonemia in obese people. The indirect estimate of glucagon secretion of 8 to 16 pmol/min in Dorset ewes in this study agreed well with the indirect estimate of 28 pmol/min in nonobese humans (49) and also with that of 26 pmol/min measured directly across the pancreas in fed sheep by Brockman (27).

Under steady state conditions, as occurred in this experiment, the basal concentrations of glucagon can be multiplied by the glucagon MCR to calculate both the estimated secretion rate (i.e., post-hepatic delivery rate) and the whole-body removal rate of glucagon. Secretion rate was calculated using the peripheral rather than the portal vein concentration of glucagon and thus the results actually represent the post-hepatic delivery rate. Plasma IRG concentrations could have been used as a surrogate for plasma glucagon in calculations of secretion rate or removal rate if the concentration of the non-glucagon immunoreactive component in plasma either is very small or was equal in concentration among different groups of animals.

Glucagon is capable of directly increasing insulin secretion and stimulating lipolysis and hepatic glycogenolysis (158). The immediate (0 to +20 min) changes in the plasma glucose and FFA concentrations after glucagon injection should reflect the direct effect

of glucagon on hepatic glycogenolysis and adipocyte lipolysis. The similar rates of increase (3.96 $mg \cdot dL^{-1} \cdot min^{-1}$) in the mean plasma glucose concentration in lean and obese sheep suggest that the hepatic responsiveness to high dose glucagon was similar in both groups of sheep; these are results agree with those in lean and obese humans (49). Because of the smaller volume of distribution of glucose in obese (~ 16 $ml \cdot 100\ g^{-1}$) than in lean (~ 21 $ml \cdot 100\ g^{-1}$) sheep (19, 105), a small difference in hepatic responsiveness to a large dose of glucagon may not have been detected in this study.

There was a rapid but transient increase in plasma FFA after glucagon injection. The initial rise likely was caused by direct glucagon stimulation of adipocyte lipolysis. The subsequent decrease to levels that approximated 50 percent of those in the basal fasted state reflect the unique ability of increased insulin to suppress hormone-stimulated lipolysis. Using normal sheep and sheep with alloxan-induced β-cell deficiency that were injected daily with insulin, Brockman (27) showed that glucagon infusion stimulated lipolysis in the absence but not in the presence of glucagon-induces rises in plasma insulin concentrations. The suppression of plasma FFA was similar in both groups of sheep in this study despite much more insulin in plasma of obese than lean sheep. Although glucagon can increase plasma FFA concentration in vivo in man and sheep (128, 141) and stimulate lipolysis in vitro (128, 137), the physiological significance of this change is unclear because the stimulatory levels of glucagon employed in those studies and this study greatly exceeded the physiological concentration of glucagon in peripheral plasma. Plasma FFA concentrations were not increased in humans whose plasma glucagon levels were increased into the high physiological level by chronic infusion of glucagon for 4 h (183).

The insulin response to glucagon stimulation was greater in obese than in lean sheep

and also in obese compared with lean humans (49). It is unclear to what degree the larger rise in plasma insulin concentration in obese than in lean sheep after glucagon injection reflected β-cell stimulation by glucagon alone or a combined stimulation by glucagon and the attendant hyperglycemia. Based on previous work (99) that determined the glucose-dose response curve for insulin secretion in lean and obese sheep, the difference in the acute insulin response between lean and obese sheep in this study was greater than that which could be attributed directly to greater responsiveness of the β-cell to glucose stimulation in obese compared with lean sheep. This suggests that obesity is associated with β-cell hyperresponsiveness to glucagon stimulation. Should this be the case, then chronic hyperglucagonemia of mild degree, as observed here in obese sheep and previously in obese diabetic and nondiabetic humans (129, 150), may play a role in maintaining exaggerated rates of insulin secretion in obese subjects.

In summary, whole-body kinetic handling of glucagon was affected minimally by dietary obesity in sheep. Obesity-associated hyperglucagonemia in fasted sheep was due to a greater entry rate of glucagon rather than a major alteration in the Vd or whole-body MCR for glucagon.

Chapter V

PLASMA MOLECULAR PROFILE OF IMMUNOREACTIVE GLUCAGON AND ISLET ALPHA CELL RESPONSE TO ARGININE IN OBESE SHEEP

INTRODUCTION

Insulin and glucagon play a major role in regulating glucose supply and utilization by body tissues. Hypersecretion of insulin and insulin resistance are common findings in obese people and animals. However, whether or not glucagon secretion is abnormal in obese-nondiabetic individuals is not clear. Obesity in humans and rodents associated with hypoglucagonemia (140, 139), hyperglucagonemia (79, 102, 127, 150) or euglucagonemia (81, 108, 129). Such conflicting results may due to inaccurate measurement of plasma glucagon concentration by RIA. RIA measurement of plasma glucagon is complicated by the presence of other proglucagon gene products and large molecular weight immunoreactive proteins in plasma. The glucagon concentration measured in plasma depends on the specificity of the antisera used (149) in the assay, and whether or not the large immunoreactive proteins had been removed prior to the RIA quantification of plasma glucagon.

On the other hand, different levels of plasma glucagon among obese individuals may reflect multiple etiologies of obesity that exist in humans (12, 44, 158). The degree and duration of obesity has been shown to affect the degree of endocrine dysfunction in

humans (12) and sheep (94).

This study used dietary obese sheep as an animal model to determine whether or not obesity is associated with glucagon dysfunction. The objectives of this study were 1) to determine if fasting hyperglucagonemia exists in obese sheep, 2) to determine if the molecular components of plasma immunoreactive glucagon differ between lean and obese sheep under basal conditions and during an arginine-stimulated condition, and 3) to determine if α cell response to the glucagon secretagogue, arginine, differed in lean and obese sheep.

MATERIALS AND METHODS

Animals

Rambouillet and Dorset ewes were used in this experiment. Experiment 1 included five lean and five dietary obese Rambouillet ewes of similar age (4 $\pm$ 1 yr). Experiment 2 included eight lean and nine dietary obese Dorset ewes aged 5 $\pm$ 0.7 yr. As described previously (102), obesity was induced by feeding ewes a hay-grain diet ad libitum; the same diet was fed at a maintenance level to lean ewes. Once static phase obesity was achieved, obese ewes were fed a maintenance intake such that intake per unit body weight ($g \cdot kg^{-1}$) was similar in lean and obese sheep. Therefore, experiments were done when lean and obese sheep were at equilibrium weight and in zero-energy balance.

Arginine Infusion

Jugular catheters (Angiocath®, Becton Dickinson, Sandy, UT) were inserted into animals at least 16 h before each arginine infusion. L-Arginine hydrochloride (Sigma,

St Louis, MO) was dissolved in warm sterile saline and filtered through a 0.45 μm membrane (Millipore, Bedford, MA). The arginine solution (3 mmol/kg) was infused (Harvard infusion pump, Cambridge, MA) into 16-h fasted animals via the jugular vein catheter at a rate of 11.25 ml/min for 8 min, after which the infusion catheter was flushed with 5 ml saline.

Blood (4 ml) samples were collected from the jugular catheter at 30, 15, and 1 min before and 10, 15, 20, 30, 60, 80, 100, 130, 190 and 240 min after the infusion started. Samples (4 ml) collected were immediately dispensed into chilled polystyrene tubes (12 x 75 mm) containing 40 μl solution of benzamidine (250 mg) and heparin (5,000 U). Additional plasma (50 ml) was collected at the -15 min and +15 min sample times. Blood samples were kept in an ice-water bath until they were centrifuged at 1,500 x g for 10 min at 4°C. After centrifugation, additional amount of the benzamidine-heparin solution (10 μl per ml of plasma) was added to the harvested plasma and samples were placed in storage (-20°C) until analyzed. All chemicals were purchased from Sigma Co., St. Louis, MO, unless otherwise stated.

IRG Response to Arginine Stimulation in Obese Rambouillet Ewes (Experiment 1)

Chromatography. Molecular profiles of plasma immunoreactive glucagon (IRG) were determined by radioimmunoassay (RIA) measurement of the IRG content in fractions obtained from gel chromatography of plasma. Twenty microliters of plasma collected before (-15 min) and during (+15 min) arginine-stimulation were lyophilized (Virtis model 10-146MR-8A, Gardiner, New York). Lyophilized samples were reconstituted with 10 ml of distilled water and loaded (8 ml) onto a Sephadex G75-120 (2.6 x 100 cm) column. Blue dextran (> 2,000 kDa) and NaI^{125} (250 Da) were added to the sample as

internal markers for the void volume (V_0) and column bed volume (V_t), respectively.

RIA buffer (pH 8.7) contained 0.05 M Trisma base, 0.01 M disodium EDTA, 15 mM sodium azide, 16 mM benzamidine hydrochloride, 0.03 M sodium chloride and 0.5% bovine serum albumin. For each chromatograph, 110 fractions (5 ml each) were collected by automatic fraction collector (Varioperpex® model 12000, LKB Pharmacia, Piscataway, NJ). A peristalic pump (S161 25, LKB Pharmacia, Piscataway, NJ) maintained a column flow rate of 15 ml per h. Fractions collected were kept frozen (-20°C) until analyzed.

The column was calibrated before the first and after the last sample was chromatographed in this experiment. The column was calibrated using the molecular markers blue dextran (> 2,000 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), myoglobin (17.8 kDa), cytochrome c (12.4 kDa), aprotinin (6.5 kDa), [^{125}I]glucagon (3.5 kDa), and [^{125}I]Na (250 Da). Eluent (pH 8.7) for column calibration was the same as the RIA buffer except that it contained 0.05 mM benzamidine and no BSA; flow rate, fraction volume and number of fractions collected were the same as for chromatography of plasma samples. The absorbance of each column fraction collected was measured at wavelength 280 nm and 620 nm to determine the elution positions of each molecular marker. Elution position was expressed by the coefficient of distribution (Kd).

IRG Response to Arginine Stimulation in Obese Dorset Ewes (Experiment 2)

Chromatography. Molecular profiles of plasma IRG in lean and obese sheep during basal (-15 min) and arginine-stimulated (+15 min) conditions were determined by gel chromatography and RIA. Plasma (-15 and +15 min) was lyophilized and stored frozen

(-20°C) until chromatographed. However, due to a sudden discontinuance of the commercial [^{125}I]glucagon supply, and the technical difficulties in preparing quality [^{125}I]glucagon (see Chapter 3), lyophilized samples and fractions collected from previously chromatogrpahed samples were stored for up to a year. Prolonged storage of lyophilized plasma or column fractions, but not plasma, resulted in loss of the large immunoreactive plasma proteins. Lyophilized samples and column fractions from 4 lean and 2 obese sheep were lost. Therefore, an additional 2 lean and 4 obese Dorset ewes were infused with arginine, and serial samples were taken as described previously.

Because of the problems due to lyophilization and the relative low resolution characteristics of Sephadex G75-120 gel, the remaining and newly collected plasma samples collected at 15 min before and 15 min after the arginine infusion were chromatographed on Sephacryl 100 high resolution gel (S100HR) packed in a 2.6 x 100 cm column (LKB Pharmacia, Piscataway, NJ). Ten to fifteen milliliters of plasma were loaded directly onto the column. The column was calibrated with blue dextran (2,000 kDa), bovine serum albumin (66 kDa), myoglobin (17.8 kDa), insulin (6 kDa), glucagon (3.5 kDa) and vitamin B_{12} (1.4 kDa). We found that NaI^{125} was not suitable for use as a bed volume marker in this column because it adhered to the gel and eluted after the bed volume. Therefore, blue dextran and vitamin B_{12} were used as internal markers for V_0 and V_s, respectively. The column as well as the fraction collector were kept at 4°C. The column buffer described in Experiment 1 was used as the eluent for column calibration and sample chromatography. Flow rate, fraction size, and number of fractions collected were as described for Experiment 1. Fractions were stored frozen (-20 °C) for less than 7 d before their IRG content was quantified by RIA.

Although S100HR gel had better resolution than the Sephadex G75-120 gel,

chromatographs from the S100HR column often showed multiple peaks at the elution position of glucagon (3500 Da) of 0.6 to 0.9 Kd. For calculation purposes, IRG measured in fractions eluting between 0.6 and 0.9 Kd were assumed to be glucagon (3500 Da). To support this assumption, sheep plasma was chromatographed and 1.5 ml of each fraction eluting at the position of glucagon (0.6 - 0.9 Kd) were pooled, lyophilized, and reconstituted with 5 ml distilled water. The concentrated eluent was loaded (5 ml) onto a Sephadex G25-150 column (1.5 x 85 cm). RIA buffer was used as column eluent, flow rate was ~20 ml/h and the fraction volume was 2 ml. Blue dextran and vitamin B_{12} were used as internal markers. Eighty fractions were collected and their immunoreactive glucagon content was determined in duplicate. This was done to determine if fractions eluting at 0.6 to 0.9 Kd on the S100HR column contained only glucagon or glucagon plus some other immunoreactive proteins.

Radioimmunoassay

Crystallized bovine glucagon (Lilly Research Laboratories, Indianapolis, IN) was used as standard. Polyclonal rabbit anti-glucagon sera, validated for the measurement of glucagon and IRG in sheep, was used in the RIA (101). The chloramine-T method (pH 7.4) was used to prepare [^{125}I]glucagon as described in Chapter 3. Glucagon antisera, [^{125}I]glucagon and goat anti-rabbit IgG were diluted using RIA buffer.

In view of the possibility that a high level of arginine may interfere in the RIA for measurement of plasma glucagon (89), standard curves were run in the absence and presence of (0.02, 0.2, 1, 2, 4 and 8 mM) arginine to check if glucagon could be quantified accurately in plasma collected during and after the arginine infusion. The maximum plasma concentration of arginine was estimated to be approximately 1 to 2 mM

in lean and obese sheep infused with 3 mmol/kg arginine. This calculation was based on the finding that plasma volume in lean and obese sheep was approximately 2.5 and 3.5 liters, respectively (94).

RIA of IRG in column fractions. Concentrations of IRG in fractions from the three different columns (Sephadex G75-120, Sephacryl 100 HR and Sephadex G25-150) were measured by the double-antibody RIA method. Assay conditions were as follows. Eight hundred microliters of column fractions or glucagon standard (3.125, 6.25, 12.5, 25, 50, 100 and 200 pg/ml) diluted in column eluent were incubated with 0.1 ml glucagon antisera (final dilution 1:60,000) at 4°C for 18 - 24 h. Fifty microliters of [^{125}I]glucagon (~20,000 CPM) were added to each assay tube; incubation was continued 18 - 24 h at 4°C before fifty microliters of goat anti-rabbit IgG (Calbiochem, San Diego, CA) diluted 1:300(id) were added. This mixture was incubated further for 48 h at 4°C. The assay was terminated by the addition of 3.8 ml cold (4°C) rinse buffer (pH 8.7; 0.05 M Trisma base, 2 mM EDTA, 15 mM sodium azide) and centrifugation at 1500 x g for 45 min at 4°C. The supernatant was discarded and the precipitate in each assay tube was counted in an automatic gamma counter for 2 min.

RIA of Plasma IRG and glucagon in PEG-extracted plasma. Plasma samples collected before and after the arginine infusion in Rambouillet and Dorset sheep were assayed directly for IRG content using the double-antibody RIA procedure. Assay conditions for plasma IRG were similar to those for the RIA of column fractions except that 200 μl of plasma or standards (12.5, 25, 50, 100, 200 and 400 pg/ml) diluted in RIA buffer were assayed. In addition, aliquots (850 μl) of each plasma sample from Dorset sheep were

extracted with polyethylene glycol (PEG) to remove large immunoreactive protein content and allow more accurate measurement of glucagon concentration (see Chapter 3). PEG-extraction was achieved by adding 150 μl of PEG-rinse buffer mixture (35% w/v) to 850 μl plasma. The plasma-PEG mixture was vortexed and centrifuged at 1,500 x g for 45 min at 4°C. The supernatant was collected and assayed for glucagon by RIA using PEG to separate antibody-bound and free [^{125}I]glucagon as described in Chapter 3.

Radioimmunoassay of insulin. Plasma samples collected serially before and after arginine infusion in Rambouillet and Dorset sheep were assayed for insulin content by solid-phase RIA (Diagnostic Product Co., Los Angeles, CA) validated for measuring plasma insulin in sheep (McCann JP, unpublished data).

Calculations and Statistical Analysis

Elution positions of the molecular markers and the immunoreactive glucagon (IRG) peaks were identified by their chromatographic coefficient of distribution (Kd), calculated as $Kd = (V_e-V_0)/(V_s-V_0)$, where V_e is the elution volume of interest, and V_0 and V_s are the elution fractions of the void volume and the column bed volume, respectively. The Kd used to identify the base-to-base positions of each IRG peak in chromatographs of individual sheep were derived from the chromatograph of data pooled within breed regardless of body condition. IRG that eluted between 0 Kd and 0.1 Kd was a protein with a molecular size $\geq$145 kDa. Other IRG peaks included a 61 kDa protein and glucagon (3.5 kDa). Quantity of IRG in each peak was calculated by multiplying the summation of IRG concentrations (pg/ml) in fractions within the designated Kd range by the fraction volume (5 ml). Total IRG for the chromatograph was determined by

summing the IRG content of each fraction (pg/ml x fraction volume) for all fractions between V_0 and V_s. The percentage of each molecular species of IRG in plasma was calculated by dividing the IRG content of each peak by the total IRG content of the chromatograph and multiplying the answer by 100.

Data from Dorset and Rambouillet groups initially were analyzed separately. The effects of body condition, arginine treatment and their interaction on the molecular profile of plasma IRG were tested. Heterogeneity of variance was present in the data from the plasma chromatograhs as determined by Bartlett's test (152). A logarithmic transformation of the data was done before statistical analysis because the coefficient of variance among each treatment groups were similar (152). The log-transformed data for the molecular profile of plasma IRG then were subjected to split-plot ANOVA for an incomplete block design (Systat Inc., Evanston, IL). Body condition contained 2 levels of treatment (lean and obese) and the arginine-dose subplot contained 2 levels of treatment (basal and arginine-stimulated). Differences among means were compared using Fisher's protested LSD test if a significant F-value ($P<0.05$) was found (152).

Differences in the plasma IRG, glucagon and insulin responses to arginine stimulation between lean and obese sheep were tested using the repeated-measures analysis of Gill (53). The acute (0 to +20 min) and total (0 to +100 min) response areas of plasma IRG, glucagon and insulin to iv arginine were determined by calculating area-under-the-curve (AUC) less basal AUC, and differences in response AUC between lean and obese sheep were tested using Student's t test. The level of probability designated as significant was $P<0.05$.

RESULTS

Body condition

Body weight, body mass index and estimated body fat differed ($P<0.05$) between lean and obese sheep (Table 9). Although the body weights of obese Dorset and obese Rambouillet ewes were similar, the degree of obesity was great in Dorset ewes as assessed by the body mass index. The lean Dorset sheep were relatively leaner than the lean Rambouillet ewes. Therefore, the lean and obese contrast in Dorset ewes was larger than that in Rambouillet ewes.

TABLE 9. *Indexes of obesity in Dorset and Rambouillet Ewes*

Body Condition		Rambouillet	Dorset
Body Weight (kg)	Lean	50 ± 2	41 ± 2*
	Obese	83 ± 3	91 ± 3
Body Mass Index (kg/cm)	Lean	0.8 ± 0.02	0.6 ± 0.01*
	Obese	1.2 ± 0.03	1.4 ± 0.04*
Estimated Body Fat (kg)	Lean	13 ± 0.8	9 ± 0.6
	Obese	28 ± 1.3	32 ± 1.6
Estimated % Body Fat	Lean	29 ± 0.7	24 ± 0.9*
	Obese	37 ± 0.5	39 ± 0.6*

All values within breed differed between lean and obese sheep ($P<0.05$). Differences ($P<0.05$) between breeds within same body condition are indicated by *. Body mass index is the ratio of body weight to wither height. Estimated body fat and % body fat were calculated according to Reid (130). Five lean and five obese Rambouillet ewes and eight lean and nine obese Dorset ewes were included in this experiment.

Experiment 1

Figure 20 shows the calibration curve for the Sephadex G75-120 (2.6 X 100 cm) column. Calibration results were similar before and after the experimental samples were chromatographed.

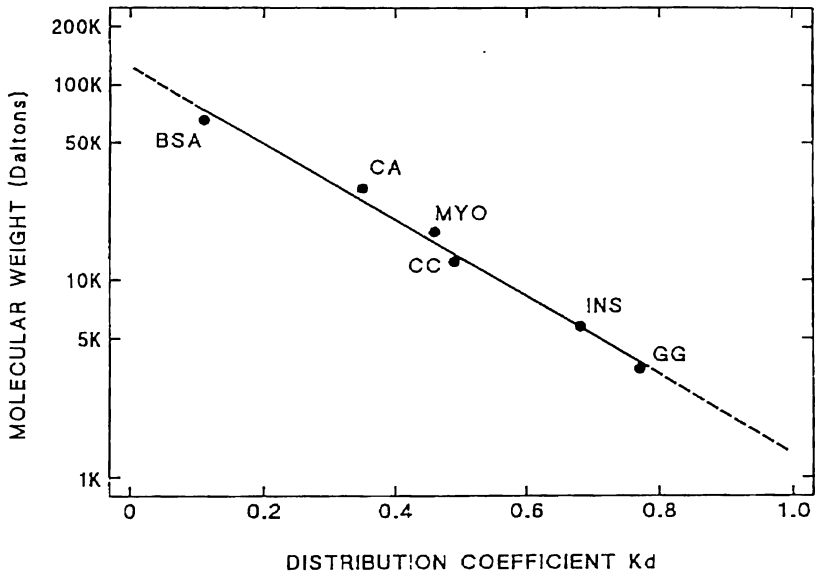

Figure 20. Sephadex G75-120 column (2.6 x 100 cm) calibration curve. Molecular markers were BSA, bovine serum albumin; CA, carbonic anhydrase; MYO, myoglobin; CC, cytochrome c; INS, insulin; GG, glucagon. Blue dextran and NaI^{125} were used as void volume and bed volume markers, respectively. Each data point was an average of 3 calibrations before and 3 calibrations after all the experimental samples had been chromatographed. The linear regression equation of all the data points is $\log Y = -1.96X + 5.09$, $r = -0.994$.

The molecular profile of plasma IRG in lean and obese sheep before (-15 min) and after (+15 min) arginine infusion (-15 and +15 min) is shown in Figure 21. Elution positions of the void volume, glucagon and bed volume were consistent for all the Rambouillet plasma samples that were chromatographed. In agreement with results reported in Chapter 3, the large molecular weight immunoreactive proteins were composed of at least two proteins. The larger one eluted at the void volume of the

column, hence it had a molecular weight of at least 123 kDa. The smaller protein eluted at 0.15 Kd and had an approximate molecular weight of 63 kDa. These two large immunoreactive proteins were observed consistently in the basal plasma samples of the both lean and obese sheep. Arginine-induced rises in the plasma levels of IRG did not affect the molecular profile of these two proteins. For the chromatographs of lyophilized samples, small amounts (< 5 pg/ml of fraction) of immunoreactive material were measured between 0.3 - 0.6 Kd. These immunoreactive proteins were affected by neither body condition nor iv arginine challenge. Glucagon (3,500 kDa) eluted consistently at approximately 0.78 Kd.

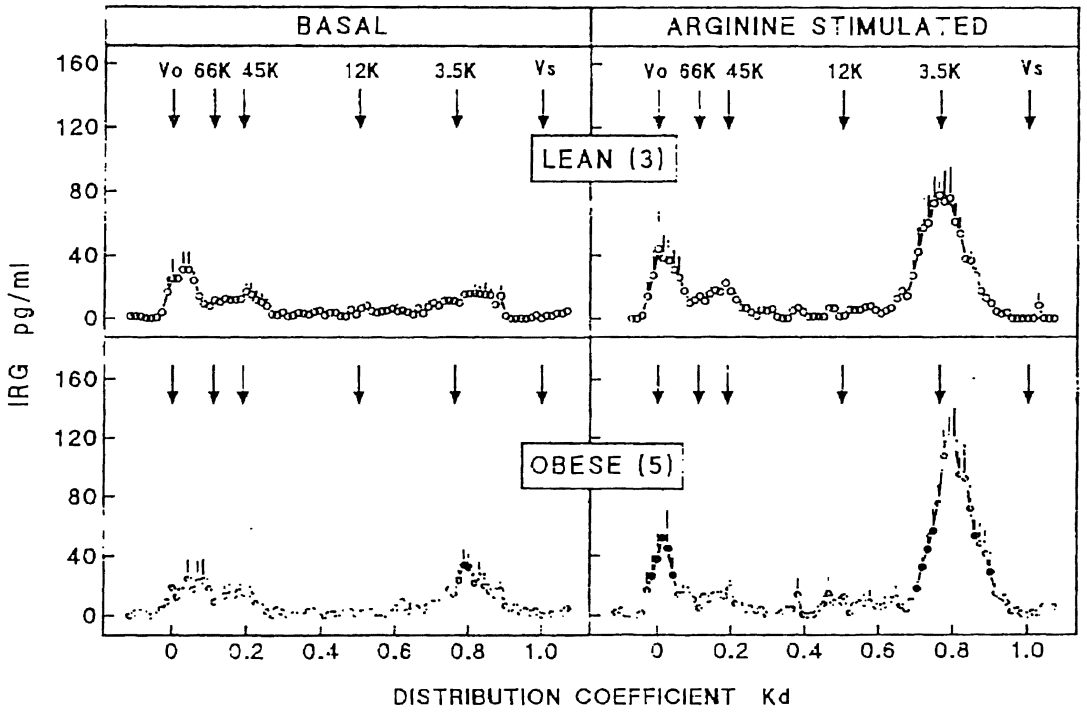

Figure 21. Molecular profile of plasma immunoreactive glucagon (IRG) in 16-h fasted lean and obese Rambouillet ewes before (basal) and during an arginine-stimulated state. Twenty milliliters of lyophilized plasma were reconstituted with 10 ml distilled water, and 8 ml of the reconstituted sample were chromatographed on Sephadex G75-120 (2.6 X 100 cm) column. Values of the y-axis are pg of IRG per ml of column fraction (5 ml per fraction).

The total quantity of glucagon and the large immunoreactive proteins present in chromatographed plasma are shown in Figure 22. The concentrations of the ≥123 kDa protein and 63 kDa protein were affected by neither body condition nor arginine stimulation; the glucagon concentration was similar ($P > 0.05$) in lean and obese sheep during the basal or arginine-stimulated conditions but the increase in glucagon following arginine infusion was significant ($P < 0.01$) in both groups.

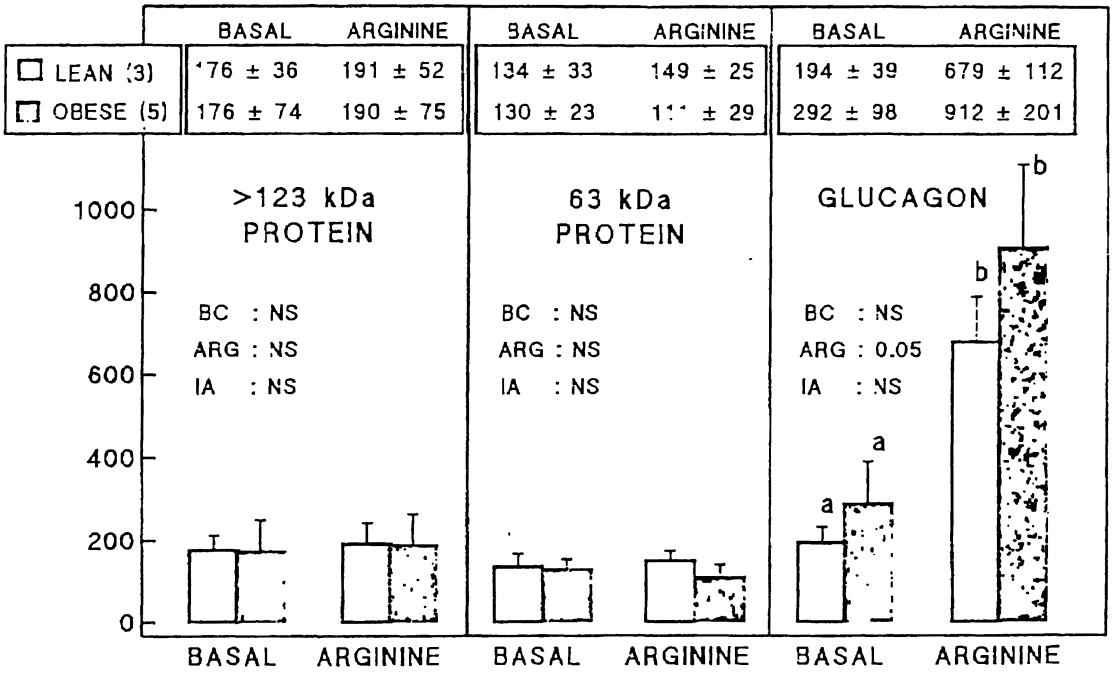

Figure 22. Quantitative illustration of the molecular species of plasma IRG in lean and obese Rambouillet ewes before (BASAL) and during the arginine-stimulated state (ARGININE). The quantity of each immunoreactive peak displayed in Figure 17 was calculated as described in Methods. Inserted table shows the values (pg per ml plasma chromatographed) for the histogram bars. The probability values for the treatment effects of body condition (BC), arginine infusion (ARG) and their interaction (IA) were derived from split-plot ANOVA of the log-transformed data. Values within a panel with different lower case letter are different (< 0.05).

The basal plasma IRG was similar ($P > 0.05$) in lean (222 ± 21 pg/ml) and obese (316 ± 88 pg/ml) sheep (Figure 23). Plasma IRG response to iv arginine stimulation was biphasic. Both groups showed a 2- to 3- fold increase in their basal plasma IRG level within 10 min after starting the arginine infusion. Plasma IRG levels remained 2 to 3 fold above basal at and 20 min after the arginine infusion began. Both groups showed a slight increase in their IRG concentration started from +40 min, but at no time did the IRG concentration in obese sheep significantly exceed that in lean sheep. Neither the acute (8.0 ± 1.4 vs 11.2 ± 5.0 $pg \cdot ml^{-1} \cdot min^{-1}$) nor the overall area (66 ± 8 vs 82 ± 26 $ng \cdot ml^{-1} \cdot min^{-1}$) for the plasma IRG response to iv arginine challenge differed ($P < 0.05$) between the lean and obese groups.

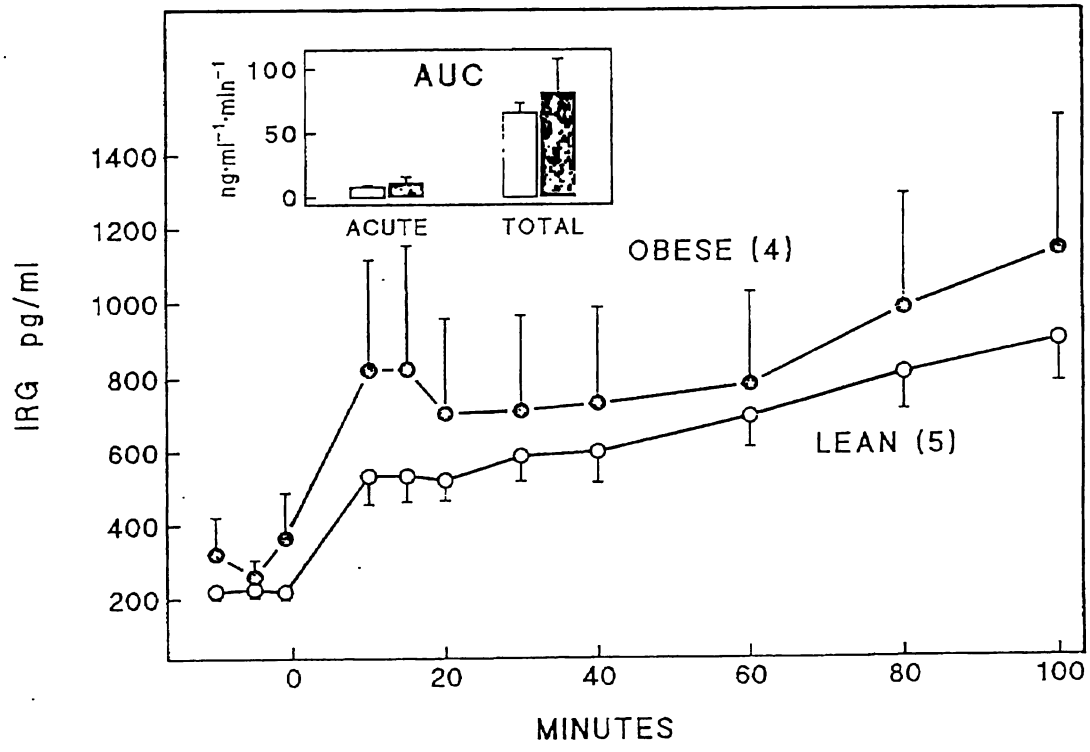

Figure 23. Plasma immunoreactive glucagon (IRG) response to iv arginine (3 $mmol \cdot kg^{-1}$) in lean and obese Rambouillet ewes. The basal plasma IRG concentration was similar ($P > 0.05$) in lean and obese sheep. Inserted diagram represents the acute (0-20 min) and total (0-100 min) areas (AUC) of the plasma IRG response to arginine stimulation in lean and obese groups.

The basal plasma insulin concentration was higher ($P < 0.05$) in obese (20 $\pm$ 8 μU/ml) than lean (4.3 $\pm$ 0.9 μU/ml) Rambouillet sheep. The plasma insulin response to arginine infusion was biphasic in both lean and obese sheep (Figure 24). Concentrations of insulin were greater ($P < 0.05$) in obese than lean sheep from 0 to +20 min, and in the period from 80 to 100 min after the arginine infusion began. The plasma insulin level exceeded ($P < 0.05$) its basal concentration in both groups at and 20 min after the arginine infusion began. Plasma insulin concentration in obese sheep continued to rise from +40 to +100 min of the experimental period. The acute response area (0.84 $\pm$ 0.25 vs 0.28 $\pm$ 0.11 mU $\cdot$ ml^{-1} $\cdot$ min^{-1}) and the overall response area (5 $\pm$ 1.6 vs 1.5 $\pm$ 0.3 mU $\cdot$ ml^{-1} $\cdot$ min^{-1}) above basal were greater ($P < 0.05$) in obese than lean sheep.

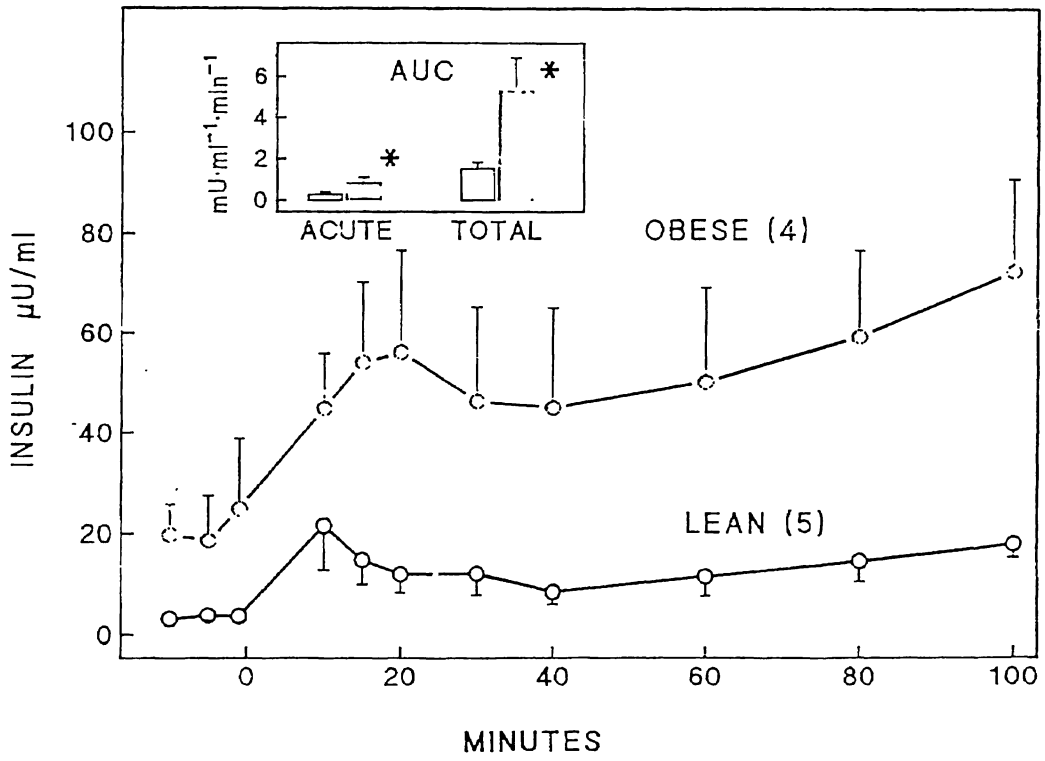

Figure 24. Plasma insulin response to iv arginine (3 mmol $\cdot$ kg^{-1}) in lean (open circles or bars) and obese (solid circles or bars) Rambouillet ewes. Basal plasma insulin concentrations were higher ($P < 0.05$) in obese than lean sheep. Inserted diagram shows acute (0-30 min) and total (0-100 min) response areas (AUC) for plasma insulin to arginine stimulation in obese than lean group.
* different from lean group, $P < 0.05$.

Experiment 2

Calibration results for the Sephacryl 100 HR (2.6 X 100 cm) column were similar before the first and after the last plasma samples were chromatographed. The combined calibration data are shown in Figure 25.

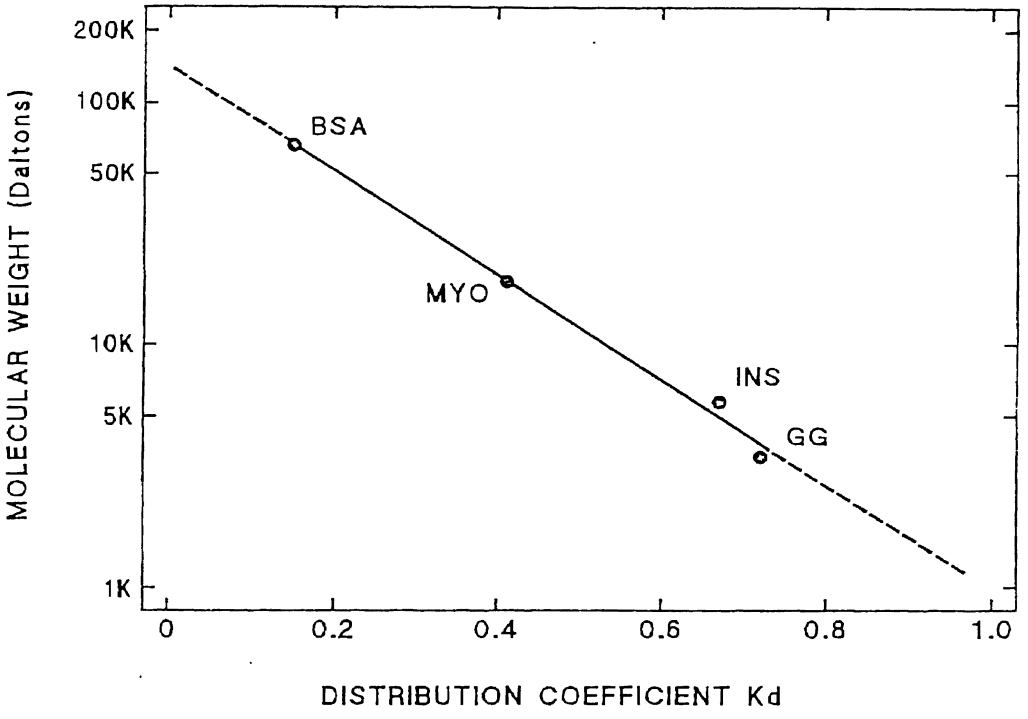

Figure 25. Sephacryl 100 HR (2.6 x 100 cm) column calibration curve. Molecular markers were: BSA, bovine serum albumin; MYO, myoglobin; INS, insulin; GG, glucagon. Blue dextran and vitamin B_{12} were used as void volume and salt peak markers, respectively. Each data point was an average of 3 calibrations before and 1 calibration after the experimental plasma samples had been chromatographed. The linear regression equation of the mean data points was
$\log Y = -2.31X + 5.16$, $r = -0.994$.

Molecular profiles of plasma IRG in lean and obese sheep are shown in Figure 26. The first protein peak eluted at the void volume and had a molecular weight of ≥ 145 kDa. The second protein peak eluted at 0.15 Kd had a molecular weight of approximately 61 kDa; presumably it apparently corresponded to the 63 kDa protein measured in Experiment 1. Small amounts of immunoreactive material eluting at 0.3 to 0.6 Kd in Experiment 1 were not observed here when non-lyophilized plasma was chromatographed.

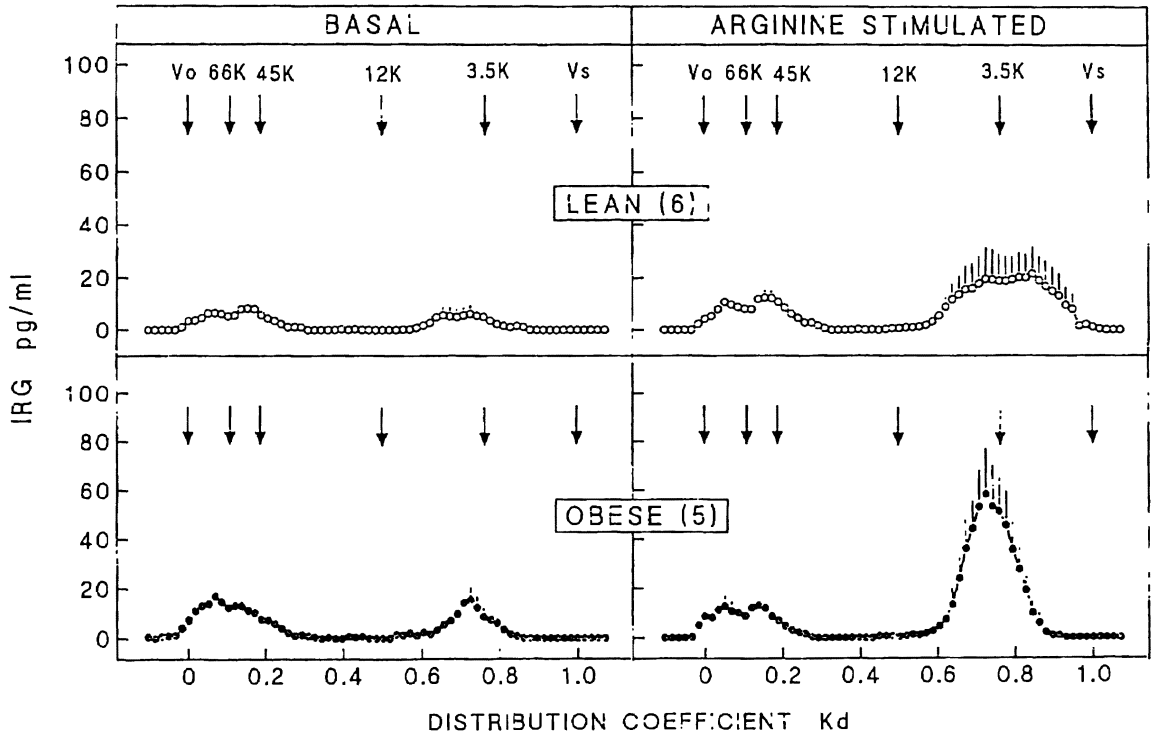

Figure 26. Molecular profile of plasma immunoreactive glucagon (IRG) in 16-h fasted lean and obese Dorset ewes before (BASAL) and during the arginine-stimulated state. Ten to fifteen milliliters of plasma were chromatographed on Sephacryl 100HR (2.6 X 100 cm) column. Values of y-axis are pg of IRG per ml of column fraction (5 ml per fraction).

As found in Experiment 1, the plasma concentrations of the ≥145 protein and 61 kDa protein were affected by neither arginine stimulation nor body condition (Figure 27). However, the basal plasma glucagon level in chromatographed plasma was higher ($P < 0.01$) in obese than lean sheep. The post-arginine plasma concentration of glucagon also was higher ($P < 0.05$) in obese than lean sheep.

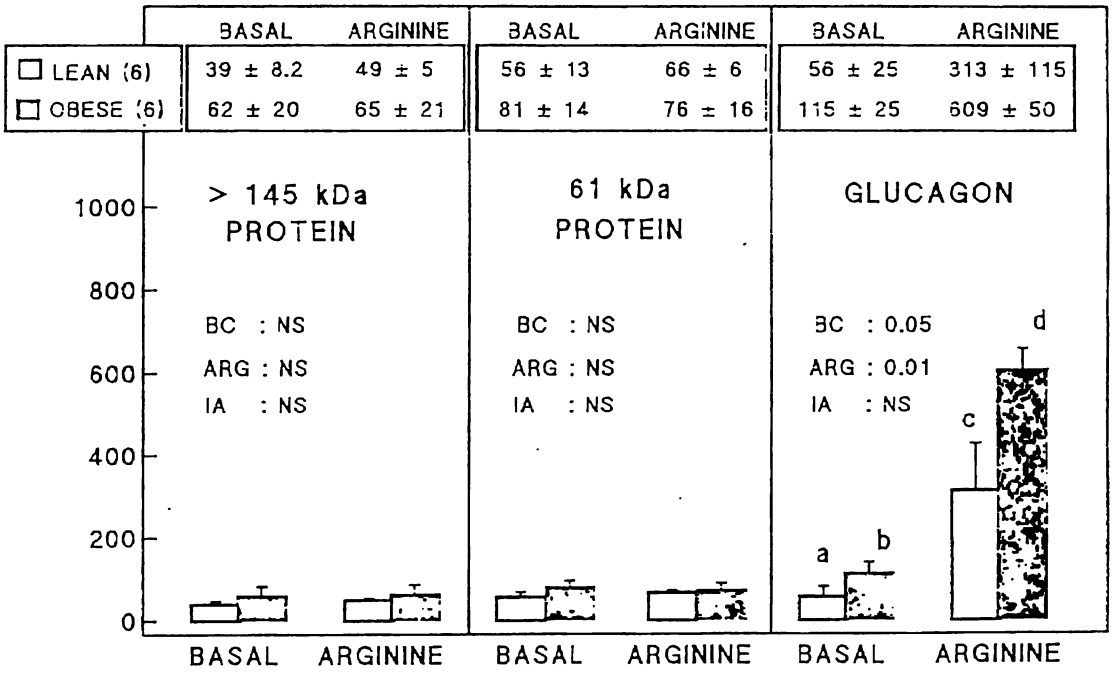

Figure 27. Quantitative illustration of the molecular species of plasma IRG in lean and obese Dorset ewes before (BASAL) and during arginine-stimulated state (ARGININE). The quantity of each immunoreactive peak displayed in Figure 22 was calculated as described in Methods. Inserted table shows values (pg per ml plasma chromatographed) for the histogram bars. The probability values for the treatment effects of body condition (BC), arginine infusion (ARG) and their interaction (IA) were derived from split-plot ANOVA of the log-transformed data. Values within a panel with different lower case letter are different (<0.05).

Basal plasma IRG tended to be higher ($P<0.1$) in obese (153.2 $\pm$ 36.26 vs 70.8 $\pm$ 5.3 pg/ml) than lean sheep. Glucagon concentrations in PEG-extracted plasma were greater ($P<0.05$) in obese (114.3 $\pm$ 31.0 pg/ml) than lean (46.7 $\pm$ 5.2 pg/ml) sheep. Plasma concentrations of IRG and glucagon before and after iv arginine stimulation were greater ($P<0.05$) in obese than lean throughout the 250 minute experimental period (Figure 28).

Plasma total IRG and glucagon increased by approximately 3 to 4 fold within 10 min after starting the arginine infusion in both groups. At and 30 min after the arginine infusion began, the plasma concentration of IRG and glucagon in obese sheep remained at values approximately four-fold higher than their basal levels. In lean sheep, however, the plasma IRG and glucagon concentration gradually returned toward basal by 100 min after starting the arginine infusion. The acute IRG (4.2 $\pm$ 1.0 vs 5.1 $\pm$ 1.0 $ng \cdot ml^{-1} \cdot min^{-1}$) and glucagon (2.7 $\pm$ 0.6 vs 4.3 $\pm$ 1.1 $ng \cdot ml^{-1} \cdot min^{-1}$) response areas to iv arginine stimulation were similar ($P>0.05$) in lean and obese sheep. The total response areas (0 to 250 min) for IRG (77 $\pm$ 16 vs 38 $\pm$ 4 $ng \cdot ml^{-1} \cdot min^{-1}$) and glucagon (64 $\pm$ 10 vs 30 $\pm$ 3 $ng \cdot ml^{-1} \cdot min^{-1}$) were greater ($P<0.05$) in obese than lean sheep.

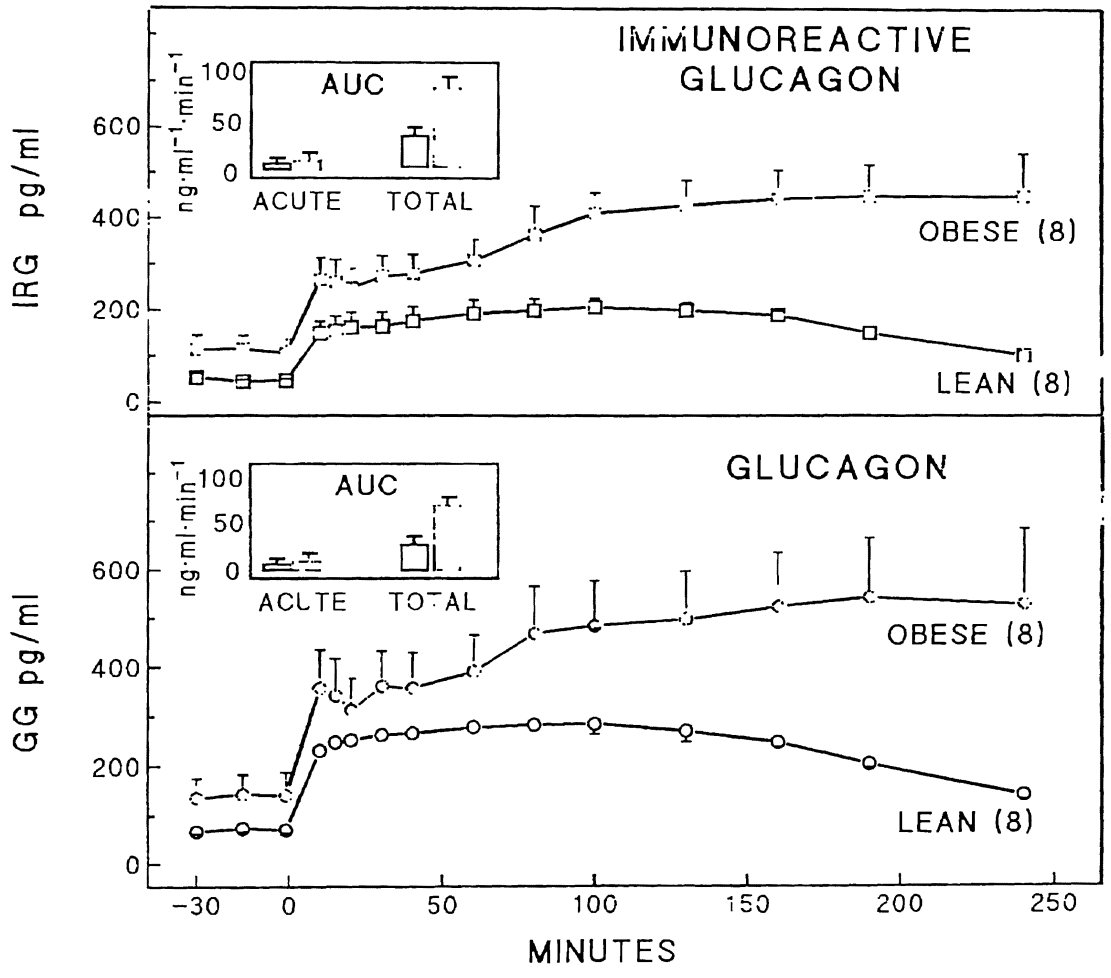

Figure 28. Plasma immunoreactive glucagon (IRG) and glucagon (GG) responses to iv arginine (3 mmol · kg^{-1}) in lean (open circles and bars) and obese (solid circles and bars) Dorset ewes. Basal plasma IRG ($P < 0.1$) and glucagon ($P < 0.05$) were higher in obese than lean sheep. Inserted diagrams represent the acute (0-20 min) and total (0-250 min) areas (AUC) above basal for plasma IRG and glucagon responses to arginine stimulation in lean and obese sheep. The parallelism of the IRG and GG concentration-time curves indicated that the constant level of [illegible] weight immunoreactive proteins remained constant through the experimental [illegible]

The basal plasma insulin concentration was higher ($P<0.05$) in obese (21.8 $\pm$ 2.5 μU/ml) than lean (6.5 $\pm$ 1.1 μU/ml) Dorset sheep. The plasma insulin response to arginine was biphasic in both lean and obese sheep (Figure 29). Plasma insulin-increased ($P<0.01$) 2- to 3-fold above its basal level in both groups by 10 min after starting the arginine infusion. At and 20 min after the arginine infusion began, the insulin level remained higher than basal in both groups but the plasma insulin concentration in the obese group was greater ($P<0.05$) than insulin levels in lean sheep from +60 to +100 min after starting the arginine infusion. The acute insulin response area was similar in obese (0.50 $\pm$ 0.15 mU $\cdot$ ml^{-1} $\cdot$ min^{-1}) and lean (0.48 $\pm$ 0.15 mU $\cdot$ ml^{-1} $\cdot$ min^{-1}) sheep. The total response area was greater ($P<0.05$) in obese (3.3 $\pm$ 1.0 mU $\cdot$ ml^{-1} $\cdot$ min^{-1}) than lean (1.8 $\pm$ 0.4 mU $\cdot$ ml^{-1} $\cdot$ min^{-1}) sheep.

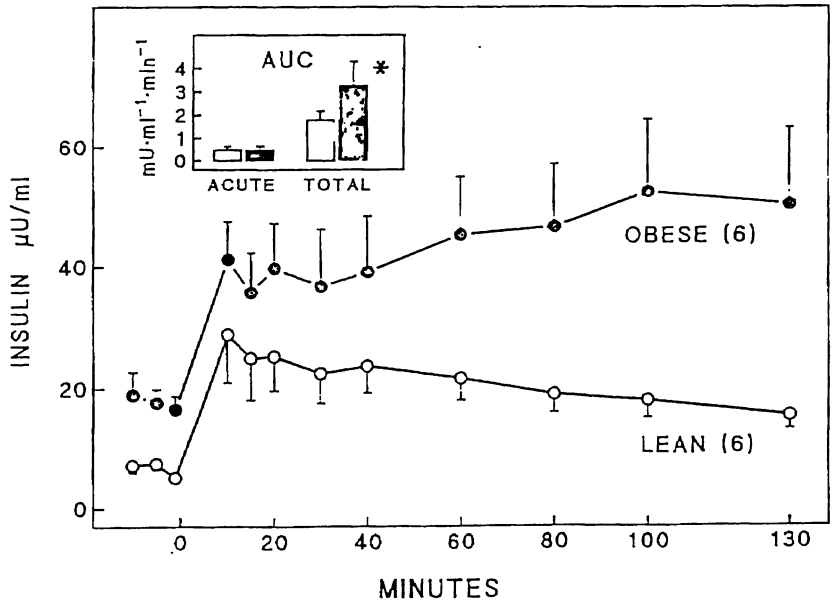

Figure 29. Plasma insulin response to iv arginine (3 mmol $\cdot$ kg $^{-1}$) in lean and obese Dorset ewes. Basal plasma insulin concentrations were greater ($P<0.05$) in obese than lean sheep. Inserted diagram shows the acute (0-20 min) and total (0-100 min) insulin response areas (AUC) to arginine stimulation in the lean and obese groups.
* different from lean group, $P<0.05$.

IRG and Glucagon in Lean and Obese Sheep

The statistical significance (Table 10) of breed, body condition, arginine infusion and their interactions on glucagon concentration were tested by 2 x 2 x 2 factorial design (Systat Inc., Evanston, IL). Although the basal and arginine-stimulated levels of plasma glucagon differed ($P<0.001$) in Rambouillet and Dorset sheep, both breeds of sheep demonstrated a similar response to arginine stimulation (Table 11).

TABLE 10. *The main and interactive effects of breed, body condition, and arginine infusion on the plasma glucagon level in sheep*

SOURCE	P
BREED	0.000
BODY CONDITION	0.013
ARGININE	0.000
BREED * BODY CONDITION	0.064
BREED * ARGININE	0.202
BODY CONDITION * ARGININE	0.709
BREED * BODY CONDITION * ARGININE	0.997

Probability values from 2 x 2 x 2 factorial ANOVA for chromatography data . Analysis was done on the log-transformed data of Experiments 1 and 2. Interactions among treatments are indicated by *.

TABLE 11. *Glucagon concentrations in Dorset and Rambouillet sheep in response to iv arginine stimulation*

	BASAL	ARGININE
Dorset (n=13)	1.71 ± 0.13	2.55 ± 0.09*
Rambouillet (n=8)	2.34 ± 0.09†	2.88 ± 0.07*†

Values are mean ± SE of log-transformed data from chromatographs.
* $P<0.001$, differ from basal;
† $P<0.01$, differ from Dorset.

In both breeds, glucagon and IRG levels always were numerically higher in obese than lean sheep. More importantly, because there was no significant interaction term involving breed, the results of Experiments 1 and 2 were pooled (breed ignored) to provide a larger data set with increased degrees of freedom for statistical analysis. The molecular profile of plasma IRG in lean and obese sheep, averaged across breeds is summarized in Figure 30.

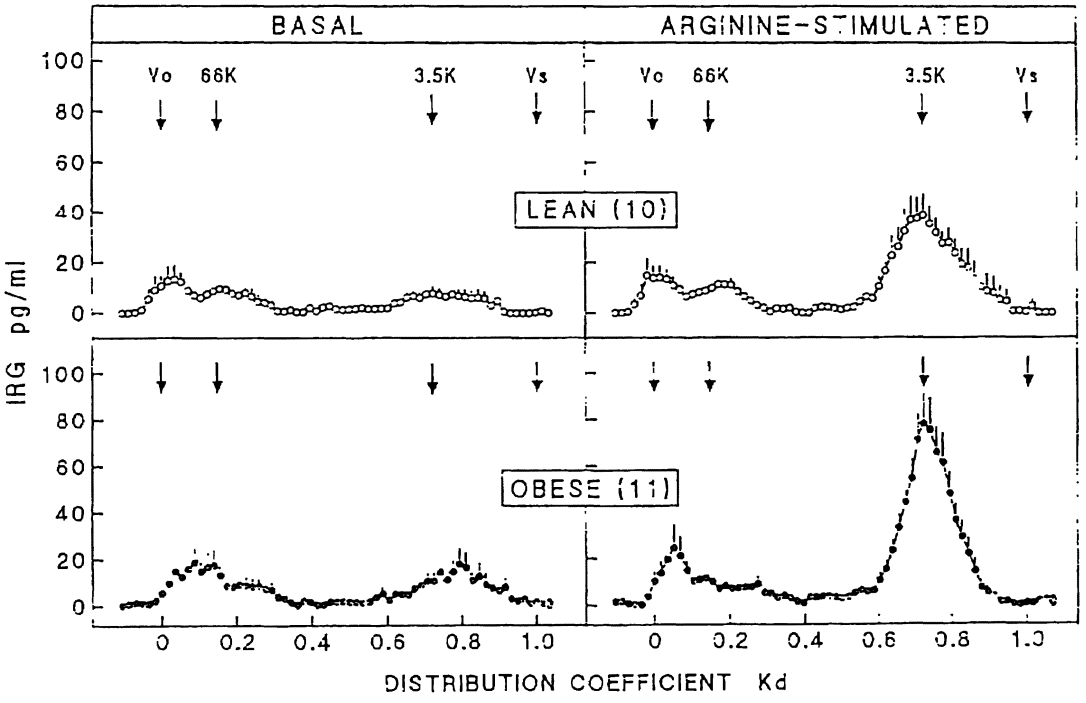

Figure 30. Molecular profiles of plasma immunoreactive glucagon (IRG) in lean (n=10) and obese (n=11) sheep before (BASAL) and during an arginine-stimulated state. Values of y-axis are pg of IRG per ml of column fraction (5 ml per fraction).

The quantity of glucagon and the large immunoreactive proteins present in chromatographed plasma are showed in Figure 31. Neither the ≥145 kDa nor the 61 kDa proteins were affected by body condition and arginine stimulation. The basal plasma glucagon concentration was greater ($P < 0.01$) in obese than lean sheep. After arginine stimulation, the plasma glucagon level was greater ($P < 0.05$) in obese than lean sheep.

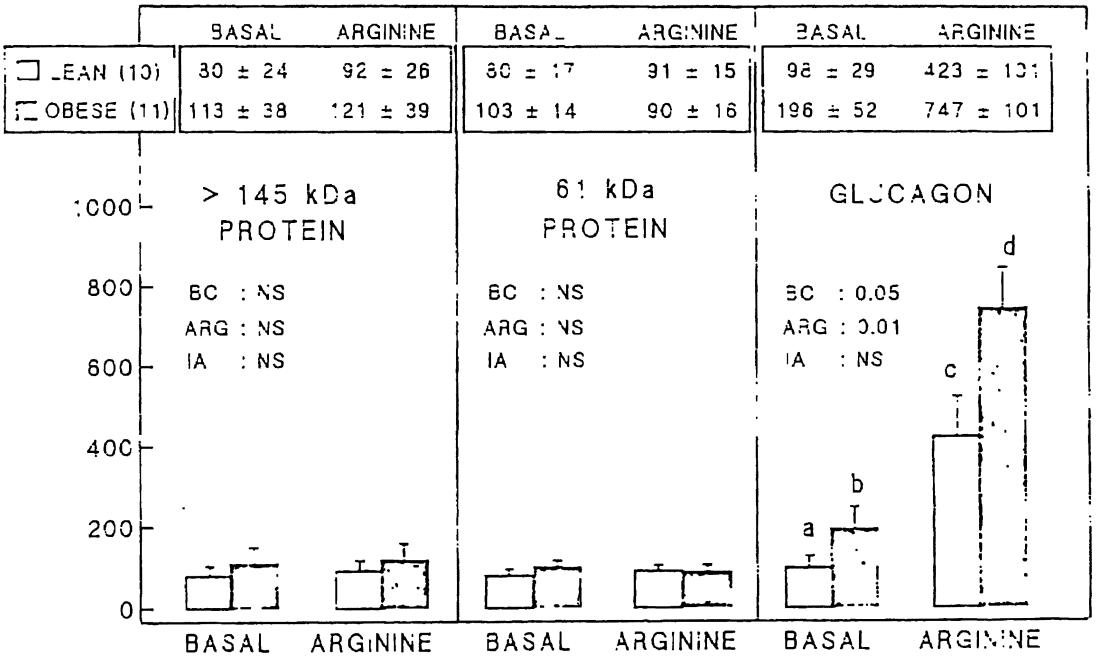

Figure 31. Quantitative illustration of the molecular species of plasma immunoreactive glucagon (IRG) in lean (n=10) and obese (n=11) sheep before (BASAL) and during an arginine-stimulated state (ARGININE). The quantity of each IRG immunoreactive peak displayed in Figure 26 was calculated as described in Methods. Inserted table shows values (pg per ml plasma chromatographed) for the histogram bars. Values within a panel with different lower case letter are different (<0.05). The probability values for the treatment effects of body condition (BC), arginine infusion (ARG) and their interaction (IA) were derived from split-plot ANOVA of the log-transformed data. Values within a panel with different lower case letter are different (<0.05).

Data for the plasma glucagon and IRG responses to iv arginine stimulation were available only from Dorset sheep (Experiment 2). Insufficient plasma was collected from Rambouillet ewes in Experiment 1 to allow measurement of glucagon in PEG-extracted plasma. Additionally, measurement of IRG in Rambouillet sheep was done only up to 100 min after arginine stimulation, whereas IRG concentrations in Dorset sheep were measured for 250 min after arginine infusion began. Therefore, data for the plasma glucagon and IRG responses to arginine stimulation in Rambouillet and Dorset sheep were not combined.

Insulin data were combined within both condition and across breed because the insulin response to arginine stimulation was not affected by an interaction between breed and body condition. The basal plasma insulin concentration was higher ($P<0.05$) in obese (19.4 $\pm$ 3.4 μU/ml) than lean (5.2 $\pm$ 0.8 μU/ml) sheep. The plasma insulin response to arginine stimulation in lean and obese sheep was biphasic (Figure 32). Basal plasma insulin levels in both groups increased (0.05) 2-fold within 10 minutes after starting the arginine infusion. At and 20 min after the arginine infusion began, the plasma insulin level remained elevated ($P<0.05$) above basal in both groups, but the plasma insulin concentration in the obese group continued to rise throughout the 100 min experimental period. Plasma concentrations of insulin were greater ($P<0.05$) in obese than lean sheep at all time points after starting the arginine infusion. The acute insulin response area to arginine infusion was similar ($P>0.05$) in obese (634 $\pm$ 136 $\mu U \cdot ml^{-1} \cdot min^{-1}$) and lean (388 $\pm$ 97 $\mu U \cdot ml^{-1} \cdot min^{-1}$) sheep. The total insulin response area was greater ($P<0.05$) in obese (2.69 $\pm$ 0.52 $mU \cdot ml^{-1} \cdot min^{-1}$) than lean (1.21 $\pm$ 0.24 $mU \cdot ml^{-1} \cdot min^{-1}$) sheep.

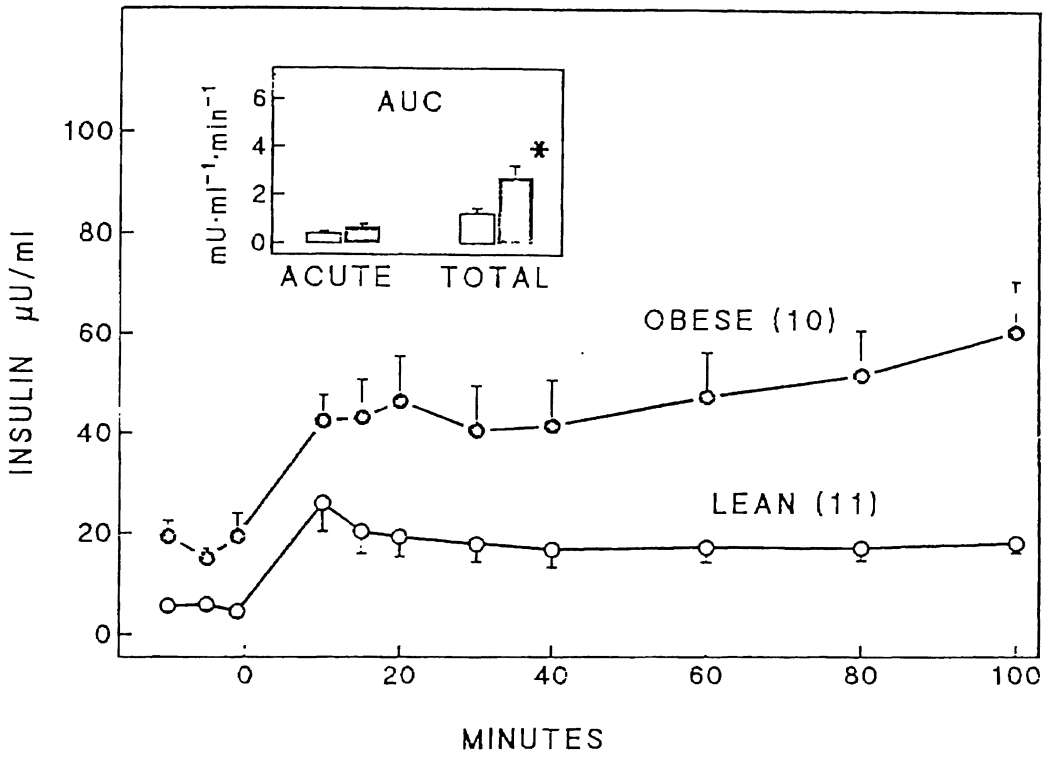

Figure 32. Plasma insulin response to iv arginine (3 mmol · kg^{-1}) in lean and obese sheep. Basal plasma insulin concentrations were greater ($P<0.05$) in obese than lean sheep. Inserted diagram shows the acute (0-20 min)and total (0-100 min) areas (AUC) for plasma insulin response to arginine stimulation in lean and obese sheep.
* different from lean group, $P<0.01$.

Interference of arginine in the glucagon RIA

Glucagon standard curves run in the absence or presence of various concentration of arginine were identical to each other (Figure 33). These results demonstrated that the presence of 0.02 to 8 mM of arginine in plasma samples would not have affected the measurement of glucagon by the RIA.

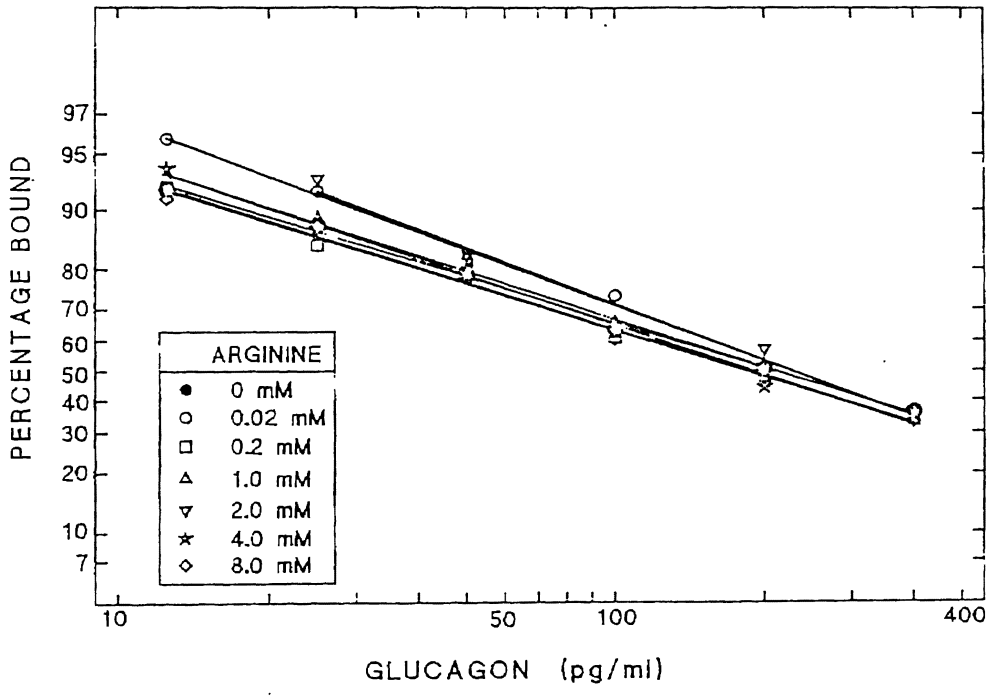

Figure 33. Binding inhibition curves for glucagon standards in the presence of 0, 0.02, 0.2, 1, 2, 4, and 8 mM arginine.

DISCUSSION

the molecular profile of plasma IRG in fasted sheep was composed of glucagon plus at least two large molecular weight immunoreactive proteins peaks which are results in agreement with those in humans (176). Several studies have detected a large immunoreactive protein with a molecular weight of 60 kDa or greater in humans (70, 79, 150, 166) and dogs (77). These researchers did not detect multiple large immunoreactive proteins because the molecular weight of the proteins exceeded the molecular range of their gel columns. This study and that by Weir (176) used a gel column with a molecular range of 1 - 145 kDa and 5 - 250 kDa, respectively. We showed that the larger immunoreactive protein had a molecular weight of ≥ 145 kDa, whereas the molecular weight of the smaller one was approximately 61 kDa. Von Schenck (170) suggested that part of the ≥ 145 kDa protein may represent gamma globulin. The chemical identity of these large immunoreactive proteins is not known.

This study found that the concentrations of the ≥ 145 kDa and the 61 kDa proteins were unaffected by dietary obesity. In agreement with results in humans (150), this study found that the amount of these two large immunoreactive proteins were variable among sheep regardless of their body condition. Based on chromatography results, the plasma glucagon concentration in obese sheep was approximately 1.8 times greater ($P < 0.01$) than that in lean sheep. The chromatographic results clearly show that the IRG difference in lean and obese sheep was due to a difference in the concentration of glucagon and not the large immunoreactive protein. Experiment 2 showed that when plasma IRG was measured directly by RIA, its concentration in obese sheep was approximately 1.9 times

higher ($P < 0.1$) than that in lean sheep. However, when plasma was extracted with polyethylene glycol to remove the large immunoreactive proteins, the glucagon value measured by RIA was approximately 2.4 times greater ($P < 0.05$) in obese than lean sheep. Failure to remove the large immunoreactive proteins before measurement of plasma glucagon by RIA masked the true difference in plasma glucagon level between lean and obese animals. This study demonstrated unequivocally that basal plasma glucagon concentration was higher in obese than in lean sheep when results were obtained using gel chromatography to separate glucagon from the large immunoreactive proteins and when plasma was extracted with PEG to remove the large immunoreactive proteins.

The function of α-cells in lean and obese sheep was evaluated in this study using arginine as a glucagon secretagogue. The acute response of glucagon to iv arginine stimulation was measured both by gel chromatography of plasma collected at 15 min after arginine infusion began and by calculating the acute (0 to +20 min) response area for the plasma glucagon concentration-time curve. Although both sets of data showed that the plasma concentration of glucagon after arginine was higher in obese than lean sheep, the incremental increase in the glucagon concentration was similar in both groups. The acute response of glucagon should reflect the arginine-induced release of glucagon stored in granules in α-cells (117). My results show that α cell responsiveness to high dose arginine stimulation was unaffected by obesity despite the observation that obesity is associated with fasting hyperglucagonemia (this Chapter) and glucagon hypersecretion in the basal state (Chapter 4).

The PEG extraction of plasma allowed an extended measurement of the glucagon response to arginine stimulation without the confounding due to the presence of the large

immunoreactive proteins. The glucagon concentration-time curves showed that the glucagon response to arginine was biphasic, which are results in agreement with those in vitro for the perfused rat pancreas stimulated with arginine (52). The second phase (+20 to +250 min) of the plasma glucagon response in this study differed between lean and obese sheep. The glucagon concentration of obese sheep increased beyond the values measured during the acute response and remained at levels 4 fold higher than those in the basal state for at least 250 min after starting the arginine infusion. The initial period (+30 to +100 min) of the second phase of the glucagon response in lean sheep was similar to that for obese sheep, but thereafter the glucagon concentration in lean sheep returned to basal values. The persistence of a high level of glucagon in the obese sheep may be due either to a slower removal rate of plasma arginine or to α-cell hyperresponsiveness to arginine stimulation in obese compared with lean sheep. Unfortunately, plasma arginine concentrations were not measured in this study. Alternatively, the prolonged increase in plasma glucagon in obese sheep may be due to a defective response of the α cells to insulin inhibition of glucagon secretion. Although the higher insulin level in obese sheep should compensate somewhat for their obesity-associated insulin resistance, the peripheral insulin concentration was increased two fold approximately during arginine stimulation in obese sheep and this increase in insulin concentration may not be strong enough to suppress glucagon release during the arginine-stimulated state. The insulin dosage necessary to suppress plasma glucagon to normal levels in obese hyperinsulinemic patients with impaired glucose tolerance was 3 to 4 times that required in normal people (63). This finding supports the concept that the persistent hypersecretion of glucagon in obese sheep may be secondary to a reduced inhibitory effect of insulin in α cells. Although the reason for the hypersecretion of

glucagon in response to arginine cannot be defined, this study and previous work (102) has clearly identified the presence of dual defects in the pancreatic islet secretion of insulin and glucagon in dietary obese sheep.

In summary, this study showed that the molecular profile of plasma IRG in both lean and obese sheep contains three immunoreactive components, a $\geq$145 kDa protein, a 61 kDa protein and glucagon (3500 Da). The plasma concentrations of the $\geq$145 and 61 kDa proteins were affected by neither obesity nor iv arginine stimulation. In addition, peripheral hyperglucagonemia and hyperinsulinemia coexist in sheep with dietary obesity. The responsiveness of α-cells to iv arginine was altered by obesity in sheep.

Chapter VI

SUMMARY AND CONCLUSIONS

Overeating causes obesity in sheep as in humans. Adult-onset diabetes mellitus is a major medical problem in many obese humans. Diabetes mellitus is a disfunction of blood glucose homeostasis. Insulin and glucagon are two major metabolic hormones that control glucose metabolism and thus the plasma glucose concentration. It is well documented that insulin metabolism is altered by obesity. However, whether or not glucagon metabolism is affected by obesity is not clearly known. Reports on the effects of obesity on glucagon metabolism in humans are conflicting.

The accurate measurement of plasma glucagon by radioimmunoassay (RIA) is a major problem in studies of glucagon metabolism in obesity. Glucagon RIA measures glucagon itself as well as some large molecular weight proteins in plasma that express glucagon-like immunoreactivity. The chemical structure and biological activities of these large immunoreactive proteins are unknown, but their presence in plasma confounds accurate measurement of small changes in plasma glucagon concentration. Therefore a plasma extraction procedure to remove the large immunoreactive proteins is an essential step prior to the measurement of plasma glucagon concentration by RIA.

In this thesis, an established animal model of dietary obesity in sheep was used to address two major research goals of 1) solving the technical problems in quantifying plasma glucagon, and 2) identifying the effects of dietary obesity on glucagon metabolism

in sheep.

To achieve the first goal, polyethylene glycol (PEG) was used to precipitate the large molecular weight proteins from plasma. The PEG-extraction method proved to be 100% efficient in removing the large immunoreactive plasma proteins and allowing 97% recovery of the glucagon in the extract. The validity of the radioimmunoassay system in measuring glucagon in PEG-extracted plasma was tested analytically in terms of sensitivity, accuracy, precision and specificity.

The second goal had three specific objectives. The first objective was to determine if the plasma glucagon concentration was greater in obese than lean sheep. This was achieved by chromatographing plasma from lean (n=11) and obese (n=10) sheep. Gel chromatography allowed separation of proteins according to molecular size. The immunoreactive glucagon (IRG) content in each of the 80 fractions collected were then measured by RIA. This generated a complete picture of plasma IRG molecular profile in lean and obese sheep. The chromatographs of lean and obese sheep plasma showed that plasma immunoreactive glucagon was composed of three major proteins: a $\geq$145 kDa protein, a 61 kDa protein, and glucagon (3.5 kDa). The plasma concentrations of the $\geq$145 kDa and the 61 kDa proteins were not different between lean and obese sheep. However, the chromatographic isolation of the glucagon component provided results which showed that peripheral plasma glucagon concentrations indeed were greater in obese than lean sheep.

The second specific objective was to test the hypothesis that the hyperglucagonemia in obese sheep was due to a greater secretion rate rather than to a slower degradation rate of glucagon. The whole body kinetics of glucagon were determined by calculating the kinetics for the plasma disappearance of a high dose (1 μg/kg) of glucagon in lean (n=8)

and obese (n=8) sheep. Blood samples were collected every 2 to 10 min for two hours after the iv injection of glucagon. Plasma samples collected were subjected to PEG-extraction then RIA measurement of glucagon. Kinetics parameters (e.g., metabolic clearance rate) were calculated and these results showed that the whole body kinetics of glucagon were altered minimally by dietary obesity in sheep. However, it was clear that glucagon secretion rate was significantly greater in obese than lean sheep.

The third specific objective was to determine if pancreatic α cell responsiveness to a glucagon secretagogue (i.e., arginine) was affected by obesity. This was achieved by infusing lean (n=11) and obese (n=10) sheep iv with arginine (3 mmol/kg). In conjunction with data from Objective 1, plasma samples collected 15 min after the arginine infusion were chromatographed and fractions assayed by RIA for their glucagon content. Chromatographs of the +15 min samples showed that arginine stimulated a significant increase in the plasma glucagon level in lean and obese sheep. However, the $\geq$145 kDa and the 61 kDa proteins in lean and obese sheep were not affected by arginine stimulation. The plasma glucagon response to iv arginine challenge was determined by measuring the plasma concentration of glucagon for 250 min after starting the arginine infusion. The overall glucagon response (250 min) to arginine was significantly greater in obese than lean sheep.

This research project validated a method for accurate measurement of plasma glucagon, and proved 1) that plasma glucagon concentrations are elevated in dietary obese sheep, 2) that the fasting hyperglucagonemia in obese sheep is due to a greater secretion rate rather than a slower degradation rate of glucagon, and 3) that islet α cells of dietary obese sheep are hyperresponsive to arginine stimulation.

Abnormal insulin metabolism in obese humans and animals is well documented, while the effects of obesity in glucagon metabolism was not clearly known. This study identified hyperglucagonemia, hyperinsulinemia, and hyperglycemia in obese sheep. The dual defects in the pancreatic islet secretion of glucagon and insulin will worsen the hyperglycemic condition that is present in obese sheep. Prolonged exposure of high level of plasma insulin, glucagon and glucose may predispose obese individuals to develop noninsulin-dependent diabetes mellitus (NIDDM).

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